A STUDY OF THE BOVINE PULMONARY RESPONSE TO *PASTEURELLA HAEMOLYTICA*. I. PULMONARY MACROPHAGE RESPONSE. II. SPECIFICITY OF IMMUNOGLOBULINS ISOLATED FROM THE BOVINE LUNG

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PREFACE

This thesis contains two chapters. These chapters were written to be complete and stand alone so that they may be submitted independently for publication in the American Journal of Veterinary Research. Preceding the first chapter is an introduction which was written to introduce the overall theme of the thesis. A summary of the overall thesis follows the references of the second chapter.

The research described here represents many hours spent trying to develop the techniques necessary to accomplish the objectives of the research proposal. There were basically three techniques that needed to be developed. These were: a method of harvesting pulmonary macrophages from the bovine lung and maintaining them in tissue culture; a method of incorporating ^{14}C labeled amino acids into *P. haemolytica*; and a method for the isolation and purification of immunoglobulins from the bovine lung.

The method described for the harvest of pulmonary macrophages is complete and should be followed easily. The method described for the incorporation of ^{14}C labeled amino acids might be further investigated to increase the percent incorporation of the isotope. The author is grateful for the advice given by Drs. Edward Grula and Jerry Merz for the success obtained with this procedure and to Dr. Calvin Beames for his cooperation in allowing me to work with a radioactive isotope in his laboratory.

The isolation and purification of immunoglobulins from the bovine

lungs was the most time consuming and exacting methodology to develop. The procedure described here provides a method for consistent recovery of pure IgA and IgG from the bovine lung. This work could not have been accomplished without the equipment, space and advice provided by Dr. Duane Garner and especially by Dr. Bruce Lessley.

The author wishes to thank Dr. R. E. Corstvet for his advice and monetary contribution to the work described herein and for his contributions in the writing of this thesis.

The author also wishes to thank Drs. Roger Panciera, Charlotte Ownby and Elizabeth Gaudy for their advice and cooperation in the preparation of this thesis.

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A special note of thanks is offered to my children, Paula and Travis, for their patience, understanding and sacrifices exhibited during the quest for the completion of this work.

The sacrifices endured and the patience and cooperation shown by my wife, Sue Ann, could never be fully acknowledged. She is what this is all about and for, and I dedicate this thesis to her.

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INTRODUCTION

Pulmonary clearance involves the continuous inactivation or removal of infectious agents from the respiratory tract.[1] The success of this pulmonary clearance is the net result of an infectious agent's ability to become established in the lung, opposed to the sum of the host defense mechanisms to neutralize and eliminate the infectious agent. Factors elicited by the host in defense of the respiratory tract include nonspecific and specific defense mechanisms.

Nonspecific mechanisms may include aerodynamic filtration, mucociliary motility and detoxification and packaging of inhaled particles by pulmonary macrophages.[1] These nonspecific defense mechanisms may be enhanced by specific defense mechanisms. Specific defense mechanisms include humoral and secretory immunoglobulins and cell mediated immune factors.

Aerodynamic filtration begins in the anterior nares and results in the deposition of particles, larger than 5 μm, in the nasopharyngeal region. Particles 2-5 μm are impinged against the epithelium in the upper airways or along the bronchial tree when the airstream abruptly alters direction.[2] Beyond the tenth bronchial division an increase in cross sectional volumes results in an abrupt reduction in airstream velocity. This reduction in air velocity results in the deposition of particles between 0.5 and 3.0 μm onto the bronchial and alveolar epithelium by gravitational sedimentation.[3] Particles less than 0.5 μm are exhaled.[3]

Particles deposited within the respiratory tract from the posterior

two thirds of the nasal cavity to the terminal bronchioles are transported to the pharynx by means of a mucociliary transport system. Transport may involve the rather simple process of mucociliary activity if deposition occurs on the bronchial tree or may engage a complex of cellular and fluid transport systems if the particles are deposited on the respiratory membrane. These pathways include 1) transport along the surface of the proximal respiratory bronchioles directly to the mucociliary apparatus of the terminal bronchiole, 2) transport in the interstitial spaces from the peripheral portion of the lobule to the mucociliary apparatus of the terminal bronchioles interstitial transport to periarteriolar, perivenous, subpleural and paraseptal positions, and 3) transport by venous and lymphatic channels to draining lymph nodes and distal reticuloendothelial organs such as liver and spleen.[2]

The precise mechanism for the transport of particles from the alveolar lumina toward the ciliated epithelium of the trachea is not yet fully understood.[5] VanAs[6] concluded that the role of mucociliary transport as a physical barrier in the airway defense mechanism may be minor. He based this conclusion on the results of his investigation which showed that there was not a continuous mucous blanket (gel layer) at any level of the bronchial tree. Instead, he found discrete particles of mucus 4 to 10 μm in diameter in the small airways. As the size of the airway increased the frequency and size of the mucus particles increased. In the main bronchi the mucus particles aggregated and were transported in streams that were as wide as 100 μm. But at no time were they continuous as previously suggested.[7,8] VanAs concluded that protection of the bronchial epithelium must involve secretory IgA, bronchial lysozyme and perhaps other proteins which reside in the fluid layer bathing the cilia.[6] Inhaled particles deposited in the region of the respiratory epithelium

initiate a complicated and poorly understood interaction between potentially infectious particles and the protein-lipid-enzyme components of the alveolar surface and the pulmonary macrophage. The pulmonary macrophage is believed to play a significant role in normal lung clearance and in the initial stages of lung infection is probably the most significant cellular defense mechanism.[9]

Specific defense mechanisms involving immunoglobulin response to respiratory tract infections have been studied extensively.[2] IgA has been found to be the primary immunoglobulin in respiratory secretions.[10] Studies with viral antigens have demonstrated that the location of instillation of the antigen predicates the location of the immunoglobulin response.[11] This response is primarily IgA.[12] If the dose of antigen is increased beyond a critical point the systemic humoral immune response becomes involved.[13]

The source of pulmonary IgA is from plasma cells located in the lamina propria beneath the epithelial cells of the airways.[14] Pulmonary IgG may arise from systemic serum IgG pools,[13] from synthesis by plasma cells in the pulmonary interstitium,[1] and possibly by pulmonary macrophages.[15]

Cell mediated immunity has been demonstrated in the respiratory tract by Burrell[16] and Henney.[17] However, due to the requirement for large numbers of viable lymphocytes and the difficulty in obtaining them, knowledge in this area of pulmonary defense is still lacking.

It is known that immune T-cells can specifically destroy cell membrane antigens against which they are sensitized. Sensitized T-cells may also enhance pulmonary macrophage phagocytic activity[1] and stimulate B-cells to produce antibodies.

In bovine shipping fever pneumonia *Pasteurella haemolytica* and

Pasteurella multocida have repeatedly been isolated from the pneumonic lesions.[18] In healthy calves these organisms are frequently found in the nasal secretion but seldom found in respiratory secretions below the larynx. However, when stressed by environmental conditions and/or concurrent viral infections, the calf may develop pneumonic pasteurellosis from which pasteurella organisms may be isolated.[18] Experimental attempts to produce the disease using the bacteria alone have, for the most part, been unsuccessful. The differences between the respiratory epithelium of the stressed calf and the normal calf is not known. However, it is known that in some instances there is an increase in respiratory secretions with a decrease in mucociliary motility, and a decrease in phagocytic activity of the pulmonary macrophage.[19]

In order to be able to produce disease a pathogenic bacterium must enter the host, multiply in host tissue, resist or at least not stimulate host defenses, and damage host tissue.[20] *P. haemolytica* is known to colonize the mucosal surface of the respiratory tract.[21] *P. haemolytica* is also known to produce an endotoxin which has dermotoxic potency comparable to that of *Escherichia coli*.[22] Gilka[23] found that *E. coli* endotoxin caused extensive pulmonary edema and inhibited pulmonary clearance of *P. haemolytica*. Panciera et al[24] have reported that they can produce resistance in the bovine lung against a subsequent challenge by exposing it to an aerosolized mist of *P. multocida*. This work has been repeated with *P. haemolytica*.

The purpose of this study was to investigate the mechanism of bovine pulmonary resistance to *Pasteurella haemolytica*. The first chapter will establish that *P. haemolytica* can be cleared from the bovine lung and that previous exposure to an aerosolized mist of *P. haemolytica* enhances the clearance rates. This chapter will also discuss *in vitro* phagocyto-

sis of *P. haemolytica* by pulmonary macrophages.

The second chapter will describe a method for the isolation and purification of immunoglobulins from the respiratory tract secretions. The specificity of these antibodies from exposed and non-exposed lungs toward *P. haemolytica* will be reported using indirect fluorescent antibody technique and an agglutination reaction.

CHAPTER I

STUDY OF BOVINE PULMONARY RESPONSE TO *PASTEURELLA HAEMOLYTICA*

I. PULMONARY MACROPHAGE RESPONSE

Summary

A resistant state was produced in the bovine lung by exposing calves to an aerosolized mist of *Pasteurella haemolytica*. Verification of the resistant state was determined by an increase in clearance of *P. haemolytica* from exposed versus non-exposed lungs over a 24 hour time period. Bovine pulmonary macrophages isolated from lung washings of calves exposed to *P. haemolytica* and non-exposed lungs failed to phagocytize *P. haemolytica in vitro*. Whole serum, pulmonary IgA and IgG from exposed and non-exposed calves did not enhance phagocytosis as measured by ^{14}C uptake or fluorescent antibody staining.

Introduction

The bovine respiratory disease complex, known as shipping fever pneumonia, has in the past resulted in substantial losses to the feedlot industry.[1] Despite the tremendous effort made to understand, prevent and/or control this disease, the economic impact it has on the cattle industry is still substantial.[1-3]

Shipping fever pneumonia is a complex disease that may be due to the interaction of environmental stress factors, viruses and bacteria that a calf may encounter during transit from the pasture or range to the feed-

lots. Stress factors encountered may include dietary changes, crowding, dehydration, lack of feed and water, chilling and inhalation of toxic substances such as diesel fuel exhaust. Viruses encountered may include infectious bovine rhinotracheitis (IBR) virus, bovine viral diarrhea (BVD) virus, bovine respiratory syncytial (RSV) virus and parainfluenza-3 (PI_3) virus.[4]

Following the initial insult to the respiratory tract by stress and/or viruses is the secondary invasion of the lung by bacteria. This insult to the lungs frequently results in a debilitated condition and/or death of the calves. The 2 bacteria most frequently isolated from pneumonic lesions are *P. multocida* and *P. haemolytica*.[5-9] Of the 2, it is thought that *P. haemolytica* is perhaps the most pathogenic.[5,9]

Pasteurella organisms have been reported to colonize bronchiolar epithelium, respiratory bronchioles, alveoli ducts and alveoli in lungs of calves that have succumbed to shipping fever pneumonia.[10] The heaviest concentration of the organisms seem to occur at the peripheries of the affected lobules. Blood vessels in the immediate area contain thrombi and excessive vascular leakage is seen in the adjacent lung parenchyma.[10]

Panciera et al[11] reported that they could produce an apparent resistant state in the bovine lung by exposing it to an aerosol mist of *P. multocida*. The resistant state was demonstrated 2 weeks after the second aerosolization of *P. multocida* when the calves were challenged by an intrapulmonary injection of *P. multocida*. Lungs that had been previously exposed to *P. multocida* exhibited small, well-defined lesions at the site of inoculation with minimal edema or no lesion at all. Lungs of control calves that were exposed to phosphate buffered saline and challenged with *P. multocida* exhibited a large focus of intense pneumonia and extensive edema of interlobular spaces in the lung surrounding the site of inocula-

tion. In some instances control calves died of acute fibrinous pneumonia and septicemic illness within 48 hours post-challenge. This work has been repeated using *P. haemolytica* as the aerosolizing and challenging agent with similar results. These studies have demonstrated that there is an immune response to pasteurella in the bovine respiratory tract. This is in agreement with the work done by Duncan[12] and Gilka.[13] Kass et al[14] in studying the clearance of *Staphylococcus aureus* and *Proteus mirabilis* from the murine lung demonstrated that the clearance of these 2 microorganisms from the murine lung was primarily due to the alveolar macrophage.

The purpose of this work was to determine if the pulmonary macrophage is a significant factor in the clearance of *P. haemolytica* from the bovine lung and if there is an increase in phagocytic activity by pulmonary macrophages of exposed calves compared to pulmonary macrophages of non-exposed calves. The effectiveness of antibodies isolated from the respiratory tract in aiding these macrophages was also investigated.

Materials and Methods

Bacteria

Media. The following bacterial media were used in this study. Brain heart infusion (BHI) agar[a] supplemented with yeast extract,[b] 1% horse serum[c] and 5% citrated bovine blood; MacConkey's agar;[a] triple sugar iron (TSI) agar;[d] SIM motility medium,[a] and tryptone broth.[a]

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[a]Difco Laboratories, Detroit, Michigan.

[b]ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio.

[c]Pel-Freez Biologicals, Inc., Rogers, Arkansas.

[d]BBL, Division Becton, Dickinson & Co., Cockeysville, Maryland.

Source of Bacteria. *Pasteurella haemolytica* isolate 12216, serotype 1 was used in this study. This isolate was originally isolated on BHI agar plates from the pneumonic lung of a calf and passaged twice in calves by intrapulmonic inoculations. After 3 passages on artificial media, *P. haemolytica* was suspended in skim milk. Five-tenth ml aliquots were lyophilized and stored at -10°C until used. Hydration of lyophilized *P. haemolytica* was with 1.0 ml of sterile distilled water.

Radioactive Labeling of *P. Haemolytica*. BHI agar plates were streaked for isolation from the hydrated lyophilized cultures of *P. haemolytica* 12216. After 24 hours incubation at 37°C in a candle jar 3 typical *P. haemolytica* colonies were picked from a plate and inoculated into one half strength BHI broth containing 0.5% horse serum. The broth suspension was incubated 12 hours at 37°C in an oscillating water bath. After incubation a 0.25 ml aliquot was used to inoculate 25 mls of the same medium. The broth suspension containing the 1% inoculum was incubated at 37°C in the oscillating water bath. After 4 hours incubation a 0.3 ml aliquot was titrated to determine the number of CFU/ml using a spot plate technique. This technique involved distributing 6 0.01 ml aliquots of each dilution on a BHI agar plate. The remaining suspension was centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant fluid was discarded. The cell pellet was resuspended in 20 mls 0.01 M PBS (Appendix), pH 7.2 and recentrifuged at 20,000 x g for 10 minutes. This procedure was repeated a second time. After the second wash the cell pellet was suspended in 25 mls of 0.0002 M glucose, 0.001 M MgCl and 30 µci ^{14}C amino acid mixture,[e] suspended in 0.01 M PBS, pH 7.2.

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[e]New England Nuclear, Boston, Massachusetts.

This suspension was incubated at 37°C in the oscillating water bath. After 60 minutes incubation, a 1.0 ml aliquot was assayed for cpm/ml to determine the total counts available. A 0.3 ml aliquot was titrated. The remaining bacteria were sedimented by centrifugation at 20,000 x g for 10 minutes at 4°C. The supernatant fluid was decanted and a 1.0 ml aliquot was assayed for cpm/ml to determine an approximate amount of isotope not incorporated. The cell pellet was resuspended in 20 mls of 0.01 M PBS, pH 7.2 by vigorous mixing. The cells were washed 4 more times in this manner. The supernatant fluid from the last wash was assayed for radioactivity. The cell pellet was resuspended in 24 mls of McCoy's 5A medium[f] supplemented with 0.03% L-glutamine v/v, titrated and assayed for radioactivity.

Coating of *P. Haemolytica* With Immunoglobulins. Washed ^{14}C labeled *P. haemolytica* suspended in McCoy's 5A (10^9 CFU/ml) was diluted 1:10 with McCoy's 5A medium. Various aliquots of this suspension were then added to an equal volume of whole serum or a specific immunoglobulin preparation prepared as described elsewhere,[15] which were used as a source of coating antibodies. These mixtures were incubated at 37°C for 10 minutes with occasional gentle shaking.

Coating of *P. haemolytica* was done with a whole serum or immunoglobulin protein concentration of approximately 1.25 mg/ml. Following the 10 minute incubation the coated bacterial suspensions were diluted 1:20 with McCoy's 5A supplemented with 0.03% L-glutamine v/v and 20% FBS.

Protein concentration determinations of sera were done using the method of Bradford[16] employing Coomassie Brilliant Blue G-250[g] as the dye

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[f]Gibco, Grand Island, New York.

[g]Bio Rad Laboratories, Richmond, California.

binding to the protein. Protein concentrations of isolated immunoglobulins were determined by absorbancy at 280 nm on a spectrophotometer[g] assuming an $E^{1\%}_{280}$ of 14.0.[17]

Experimental Animals

Calves to be used in these experiments were obtained from local beef herds and transported by truck to our research facilities. These calves were fed a maintenance diet of approximately 2-3 pounds of a 10% protein grain mix per calf and unlimited prairie hay and water.

Bacteriological Sampling of Experimental Animals

Nasal and tracheal swabs were taken within 1 week prior to the initiation of the aerosolization procedures. Three days after each aerosolization and 10 days after the second aerosolization nasal and tracheal swabs were again taken. Tracheal swabs were taken using the method of Corstvet et al.[18] This involved passing a guarded culture instrument[h] through the oral cavity into the anterior trachea. Swabs were processed for bacterial isolation by inoculating BHI agar plates. After 24 hours incubation at 37°C in a candle jar isolated colonies were picked and streaked for isolation onto BHI agar plates. After 24 hours incubation at 37°C in a candle jar isolated *P. haemolytica* colonies were identified using the following parameters: colonial morphology exhibiting hemolysis; Gram staining characteristics (Gram negative pleomorphic rod); growth on fresh MacConkey's agar plates; and acid/acid TSI slant at 24 hours; no indole production from tryptone broth after 48 hours incubation; non-motile in SIM motility media; catalase positive and oxidase negative.

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[h]Kalayjian Industries, Long Beach, California.

At the time of the first and final swabbings a blood sample was taken from each calf. The blood clot was allowed to develop at room temperature. After 24 hours serum was harvested and used for various serological procedures. These included agglutination reaction against *P. haemolytica* and a serological profile against virus associated with bovine respiratory disease, namely infectious bovine rhinotracheitis (IBR) virus, bovine viral diarrhea (BVD) virus, parainfluenza type 3 (PI_3) virus, and respiratory syncytial virus (RSV). Serology for the bovine viral respiratory disease profile was performed at the Oklahoma Animal Disease Diagnostic Laboratory. The agglutination reaction for agglutinating antibodies against *P. haemolytica* involved a slide agglutination test where 1 *P. haemolytica* colony was mixed with 1 drop of whole serum. Reactions were recorded as positive when agglutination occurred within 15 seconds.

Aerosolization of *Pasteurella Haemolytica*

A lyophilized stock culture (No. 12216) of *P. haemolytica* was hydrated with 1.0 ml of sterile distilled water. After the second passage on BHI agar plates this suspension was used to inoculate BHI agar plates (1 plate/80 mls of aerosol suspension needed). After 18-20 hours incubation at 37°C in a candle jar 10-12 typical *P. haemolytica* colonies were picked per plate and suspended in 1.0 ml 0.01 M PBS, pH 7.2. From each suspension 8 BHI plates were heavily inoculated. These plates were incubated 18-20 hours at 37°C in a candle jar. Each bacterial suspension was also used to inoculate a MacConkey's agar plate, SIM, tryptone, and TSI agar slant medium. *P. haemolytica* suspensions were made from BHI agar plates by removing the organisms from the plates and suspending them in 0.01 M PBS, pH 7.2, to an approximate concentration of 1.0×10^9 CFU/ml.

Exposure of the calf lung to *P. haemolytica* was accomplished by aerosolizing *P. haemolytica* suspension with an ultrasonic nebulizer.[i] The aerosolized mist was delivered to a 10 gallon plastic bag which had been sealed over the muzzle of the calves with foam rubber and tape.[j] The bag was also connected to a vacuum pump via a prefilter filter system[k] which served to exhaust the bag.

Aerosolization of *P. haemolytica* was conducted for 15 minutes. Fifteen second impinger samples were taken at 13 minutes with glass impingers[l] containing 20 mls of BHI broth with 1-2 drops of antifoam agent.[m] On the day of aerosolization and for 3 consecutive days thereafter the temperature and physical condition of the calves were recorded. The calves were aerosoled 2 times at a 1 week interval.

Pulmonary Clearance of *Pasteurella Haemolytica*

Eight experimental beef bred calves (300-350 pounds) were used. These calves were designated by the numbers 501-504, 506-509. Four of these calves were exposed to an aerosol mist of *P. haemolytica*. The other 4 calves were exposed to an aerosol mist of 0.01 M PBS, pH 7.2, at the same time intervals. Two weeks after the second aerosolization all 8 calves were exposed to *P. haemolytica* via aerosolization. At 0, 4, 8 and 24 hour time intervals 1 calf previously exposed to *P. haemolytica* and 1 calf exposed to PBS were killed. Death was by administration of 3.0 mls

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[i] DeVilbiss Company, Somerset, Pennsylvania.

[j] Pitman Moore, Arlington, Texas.

[k] Kewaunee Scientific Equipment, Adrian, Michigan.

[l] Ace Glass Company, Vineland, New Jersey.

[m] Sigma Chemical Company, St. Louis, Missouri.

of Rompun[n] to tranquilize followed by pithing and exsanguination. The trachea was plugged immediately after exsanguination. The lungs were removed asceptically. A 2 to 3 gram piece of tissue was removed from each lobe of each lung. These tissues were homogenized in 0.01 M PBS, pH 7.2, using a mortar and pestle. Sterile silica sand was used as an abrasive. Homogenates were diluted to 10^{-5} using 10-fold serial dilutions. Each dilution was titrated for the number of CFU/dilution using a spot plate technique. After 24 hours incubation the number of colonies per spot were counted and the number of CFU/gm of tissue calculated.

Harvest of Pulmonary Macrophages

Calves used in this set of experiments were designated LW8-LW11. Calf LW8 was exposed to an aerosolized mist of 0.01 M PBS, pH 7.2, 2 times at a 1 week interval. Lungs from calf LW9 were obtained from a local abattoir on the day it was killed. Calves LW10 and LW11 were exposed to an aerosolized mist of *P. haemolytica*. Two weeks after the second aerosol exposure the calves were killed by a blow to the head followed by exsanguination. (One calf, LW11, was tranquilized with Rompun, pithed and then exsanguinated.) Following exsanguination the trachea was plugged at the laryngeal aperature with a sterile cotton plug and the lungs and trachea were carefully removed as soon as possible. The lungs were placed in a plastic bag and transported to the laboratory on ice in an insulated container. Washing of the lungs was initiated within 45 minutes after death.

Pulmonary lavage was performed by inserting a sterile endotracheal tube, Portex 10.5[o] into the trachea and securing it in place with the

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[n]Rompun, Haver Lockhart, Shawnee, Kansas.

[o]Smith Industries, Welmington, Massachusetts.

distal end just above the branching of the right apical bronchis. A neoprene "Y" was attached to the proximal end of the endotracheal tube.

One arm of the "Y" was connected to a 2 L Erlenmeyer flask with Tygon flexible plastic tubing. The flask was fitted with a rubber stopper through which 2 glass tubes were placed. One extended from just above the stopper to the bottom of the flask and the other from just above the stopper to just below it. The Tygon tubing was connected to the longer tube. The shorter tube was connected to a pressure pump.

The second arm of the neoprene "Y" was attached with Tygon tubing to a 2 L siliconized filtering flask. (The flask was siliconized with Siliclad.[p]) This flask was fitted with a rubber stopper through which one glass tube was placed. The Tygon tubing was attached to this tube. The side arm of the flask was connected to the vacuum pump.

A modification of Fox's[19] method was used to remove macrophages from the lung. This involved gently filling the lung with sterile Rinaldi[20] salt solution (R-saline) containing 0.2% versene[q] (Appendix). This was carried out under 2 PSI. After instillation of approximately 1200-1500 mls of this solution the tubing was clamped off, the lungs were gently massaged and the fluid was removed by applying negative pressure at 2 PSI. The washings were collected into the siliconized filter flask.

The lungs were lavaged 3 times. A total of 2.0-2.5 L of washings were harvested which represented an approximate 50% recovery rate. A 50 ml aliquct was centrifuged at 500 x g for 10 minutes. The supernatant fluid was decanted. The pellet was resuspended in 2.5 mls of 40% ethyl

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[p]Siliclad, Clay Adams Div. of Becton, Dickerson & Co., Parsippany, New Jersey.

[q]Fisher Scientific, Dallas, Texas.

alcohol. This suspension was centrifuged at 500 x g for 10 minutes at 4°C. The resultant supernatant fluid was added to the 2.0-2.5 L supernatant fluid. Smears were made from the pellet which were stained with Sano stain to determine cell population. The remaining 2.0-2.5 L cell suspension was centrifuged at 500 x g for 10 minutes at 22°C. The supernatant fluid was decanted and used as the source of pulmonary secretions for the isolation of immunoglobulins as described in a companion paper.[15] The cell pellet was resuspended in a total of 200 mls of R-saline with 0.2% versene and centrifuged at 500 x g for 10 minutes at 25°C. The supernatant fluid was discarded and the cells were gently resuspended in 10 mls of McCoy's 5A medium supplemented with 0.03% v/v L-glutamine. This suspension was centrifuged at 500 x g for 10 minutes at 25°C. The supernatant fluid was discarded and the cells were again resuspended in 10 mls of McCoy's 5A medium. A 0.1 ml aliquot of the cell suspension was diluted in 9.9 mls of McCoy's 5A medium and counted in a hemacytometer. The undiluted cell suspension was then diluted to 5.0×10^5 to 1.0×10^6 cells per ml with McCoy's 5A supplemented with 0.03% v/v L-glutamine, 20% FBS,[r] 100 µg/ml gentamicin[s] and 2.5 µg/ml of amphotericin B.[t] The pulmonary macrophages were cultured in 4-cell tissue culture chambers[u] by inoculating 1 ml of the suspension into each chamber and incubating in a moist atmosphere with 5% CO_2 at 37°C. After 6 hours incubation medium with unattached cells were decanted. Fresh McCoy's 5A medium containing 0.03%

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[r]FBS, Flow Laboratories, Rockville, Maryland.

[s]Gentamicin, Schering Corporation, Kenilworth, New Jersey.

[t]Amphotericin B, E. R. Squibb & Sons, Princeton, New Jersey.

[u]No. 4804 4-cell tissue culture chambers, Lab Tek, Division of Miles Laboratories, Naperville, Illinois.

v/v L-glutamine and 20% FBS, but without antibiotics, was added to the cell monolayer and incubated approximately 18 hours as before.

Bacterial Uptake By Pulmonary Macrophages

One ml aliquots of the coated and non-coated ^{14}C labeled *P. haemolytica* were added to the macrophage monolayer. This represented a ratio of 10:1 - 100:1 bacteria/macrophage. An additional set of wells in each study was incubated with zymosan[v] to measure phagocytic index. Zymosan was used according to the method of Siraganian.[21] A second set of wells from each assay was incubated with acridine orange.[m] Cell viability was tested with acridine orange. Green fluorescing cells were scored as viable and red fluorescing cells as non-viable, when observed with a fluorescent microscope[w] using a 100-W mercury vapor lamp, barrier filter 50 (orange), and FITC excitatory filter (495 nm).[w] After inoculation the chamber slide wells were incubated at 37°C in a CO_2 environment with occasional shaking. After 30 minutes of incubation the supernatant fluids were decanted and the cell monolayers were washed with warm McCoy's 5A supplemented with 20% FBS. The cells were lysed by the addition of cold (4°C) distilled water. (Slides incubated with zymosan and acridine orange were fixed with 10% buffered formalin.) Zymosan slides were subsequently stained with PAS stain. Acridine orange slides were air dried and mounted with Eukitt.[x] Lysed macrophages were scraped from the glass with a plastic tube. A 1.0 ml sample was aspirated and dispensed into a disposable

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[v]ICN Pharmaceuticals, Inc., Cleveland, Ohio.

[w]Zeiss, Carl Zeiss, West Germany.

[x]Calibrated Instrument, Inc., Ardsley, New York.

scintillation vial.[y] Ten mls of Insta-gel[z] scintillation fluid was added and the samples were counted for ^{14}C activity in a liquid scintillation spectrometer.[z] Vials were counted for 1 minute, counting efficiency was 83%.

In the final trial phagocytic study (LW11) 2 additional slides were run for all samples. One set was incubated for 30 minutes and the second for 60 minutes. Phagocytosis was terminated by washing the monolayers with warm McCoy's 5A supplemented with 20% FBS and fixing the cells with acetone. These cells were prepared for fluorescent antibody examination using the method of Potgieter.[22] In this method the slides were flooded with anti-*P. haemolytica* immunoglobulin conjugates prepared according to the method of Corstvet et al.[23] The conjugated antiserum was diluted 1:8 in 0.01 M PBS, pH 7.2, and adsorbed with pelleted lung homogenate for 4 hours at 4°C. The conjugate was clarified by centrifugation at 20,000 x g and filtered through a 450 nm membrane filter.[aa] The flooded slides were incubated in a humid chamber at 37°C. After 30 minutes incubation the slides were washed for 20 minutes with 3 changes of PBS in a Coplin jar. The preparations were dipped in distilled water and allowed to dry at room temperature and then mounted with Eukitt.

Results

A growth curve of *P. haemolytica* using the methods described in radioactive labeling of *P. haemolytica* indicated that at the end of a 4 hour

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[y]Scientific Products, Grand Prairie, Texas.

[z]Packard Instrument Co., Downers Grove, Illinois.

[aa]Millipore Filter Corporation, Bedford, Massachusetts.

time period *P. haemolytica* was at the end of its log phase of growth. As it was desirous to maintain a viable culture this time period was used for tagging *P. haemolytica* with ^{14}C. Using this procedure approximately 35% of the isotope was taken up. Isotope counts from the fifth wash indicated that better than 95% of the isotope was associated with the bacterial cells. This represented 4.0×10^5 cpm/ml of bacterial suspension which contained 1.5×10^9 CFU/ml or approximately 3.1×10^2 CFU/cpm.

Coating of *P. Haemolytica* with Immunoglobulins

Sera used from both exposed and non-exposed calves were seen to coat *P. haemolytica* using the indirect fluorescent antibody technique (IFAT). The IgA isolated from the lung washing of non-exposed calves was not observed coating *P. haemolytica*. The IgG fraction from the non-exposed and both the IgA and IgG fractions from the exposed calf did coat *P. haemolytica*.

Pulmonary Clearance of *Pasteurella Haemolytica*

Three days after the first aerosolization *P. haemolytica* was isolated from 4 of the 8 calves used in the clearance study. Three of these calves had been experimentally exposed to *P. haemolytica*. *P. haemolytica* isolates were from the nasal and tracheal passage of 2 of these calves and nasal passage of the third exposed calf. *P. haemolytica* was also isolated from the nasal passage of 1 PBS calf. This represented the only *P. haemolytica* isolated from the nasal and tracheal passage of these calves.

A *P. haemolytica* suspension of 1.6×10^9 CFU/ml was used in the first aerosolization procedure in this experiment. Impinger samples taken during the aerosolization of each calf yielded 4.5×10^4, 7.0×10^4, 3.1×10^4,

and 7.0 x 10^4 CFU/ml for calves 501, 503, 507 and 508 respectively. For the second exposure to *P. haemolytica* a working suspension of 1.0 x 10^9 CFU/ml was used. Impinger samples taken during this aerosolization procedure yielded 1.0 x 10^5, 4.2 x 10^4, 1.7 x 10^5 and 1.9 x 10^5 CFU/ml for calves 501, 503, 507 and 508 respectively. For the final aerosolization of each calf with *P. haemolytica* a suspension of 1.6 x 10^9 CFU/ml was used. The recovery of *P. haemolytica* from the impinger sampling of these aerosolizations were 4.6 x 10^4 and 4.1 x 10^4 CFU/ml for the 0 hour calves 502 and 501 respectively. For the 4 hour calves the impinger sample values were 7.0 x 10^3 and 1.6 x 10^4 CFU/ml for calves 506 and 503 respectively. For the 8 hour calves the impinger values were 3.1 x 10^4 and 9.6 x 10^3 for calves 504 and 508 respectively. The impinger sampling of the aerosolization of the 24 hour calves yielded 1.2 x 10^5 and 5.5 x 10^4 CFU/ml respectively for calves 509 and 507.

Serological analysis of these calves showed agglutinating antibodies against *P. haemolytica* in the calves exposed to *P. haemolytica* 10 days after the second aerosolization procedure. None of the non-exposed calves demonstrated agglutinating antibodies. Serological studies also revealed that calves in both the exposed and non-exposed groups had a current or recent BVD viral infection. This was indicated by titers greater than a screening titer of 1:20 in initial and final serum samples submitted to the Oklahoma Animal Disease Diagnostic Laboratory. Calf 509 was the only calf that did not have a BVD titer at the time of the initial bleeding. This had changed to a titer greater than 1:20 by the time of the final bleeding.

The calves exposed to *P. haemolytica* exhibited an increase in temperature following the first aerosolization procedure. These temperatures ranged from 105.4°F for calf 507 to 106.8°F for calf 501. This was com-

pared to a temperature range of 101.6°F to 102.4°F for the non-exposed calves. By the fourth day following the first aerosolization the temperatures of all calves, except 501, had returned to a normal range. The temperature of this calf remained elevated (104.6°F to 107.2°F) for the duration of the experiment. This calf also became lame in one leg and was depressed. Except for calf 501 there were no elevated temperatures following the second aerosolization procedure.

Recovery of *P. haemolytica* from the lung of the 0 hour calves indicated a uniform deposition of the bacteria in each lobe of the lung for both calves; ruminal content contamination of the surface of the right apical lobe prevented sampling of it (Table 1). The average number of CFU recovered per gram of tissue from the PBS calf was 6.5×10^4. For the exposed calf the average was 6.3×10^4. Samples taken at 4 hours post-aerosolization showed a difference in clearance of viable *P. haemolytica* (Table 2) with an average of 4.7×10^4 CFU/gm of tissue being recovered from the PBS calf compared to an average of 1.4×10^4 for the exposed calf, with no organisms being recovered from a lobe of the experimental calf. At 8 hours post-aerosol the difference was even more pronounced (Table 3). Viable *P. haemolytica* was recovered from 5 of the 7 lobes of the non-exposed calf for an average of 9.2×10^2 compared to recovery of *P. haemolytica* from 2 of 7 lobes of the exposed calf for an average of 4.1×10^2 CFU/gm of tissue. At 24 hours there was no *P. haemolytica* isolated from any lobe of the exposed calf (Table 4). However, 4 of the 7 lobes of the non-exposed calf still had viable *P. haemolytica* within their parenchyma, (mean 3.75×10^3). The lungs of this calf were moist and heavy and exuded fluid from cut surface. Isolated individual lobules were deep red.

TABLE 1 -- Number of *Pasteurella haemolytica* Recovered From Calf Lungs at Zero Hour Post-aerosol Exposure

Lung Site	Non-exposed Calf (502)* (CFU/gm of Tissue)	Exposed Calf (501)** (CFU/gm of Tissue)
Left apical	3.9×10^4	3.7×10^4
Left cardiac	1.1×10^4	1.3×10^5
Left diaphragmatic	4.0×10^4	3.2×10^4
Right apical	1.0×10^4	N.D.***
Right cardiac	3.1×10^5	1.2×10^4
Right intermediate	1.8×10^4	4.2×10^4
Right diaphragmatic	2.3×10^4	1.1×10^5

*Impinger sample yielded 4.6×10^4 CFU/ml.

**Impinger sample yielded 4.1×10^4 CFU/ml.

***N.D. - Not done.

TABLE 2 -- Number of *Pasteurella haemolytica* Recovered From Calf Lungs at Four Hours Post-aerosol Exposure

Lung Site	Non-exposed Calf (506)* (CFU/gm of Tissue)	Exposed Calf (503)** (CFU/gm of Tissue)
Left apical	4.0×10^3	1.8×10^3
Left cardiac	2.3×10^3	9.8×10^4
Left diaphragmatic	4.3×10^3	5.0×10^2
Right apical	3.0×10^5	2.0×10^2
Right cardiac	5.7×10^3	2.0×10^2
Right intermediate	5.2×10^3	$<1.0 \times 10^{2}$***
Right diaphragmatic	6.0×10^3	2.0×10^2

*Impinger sample yielded 1.2×10^5 CFU/ml.

**Impinger sample yielded 5.5×10^4 CFU/ml.

***Sampling procedure could not detect *P. haemolytica* at a concentration of less than 1.0×10^2 CFU/gm of tissue.

TABLE 3 -- Number of *Pasteurella haemolytica* Recovered From Calf Lungs at Eight Hours Post-aerosol Exposure

Lung Site	Non-exposed Calf (508)* (CFU/gm of Tissue)	Exposed Calf (504)** (CFU/gm of Tissue)
Left apical	5.0×10^2	$<1.0 \times 10^{2***}$
Left cardiac	$<1.0 \times 10^2$	$<1.0 \times 10^2$
Left diaphragmatic	$<1.0 \times 10^2$	$<1.0 \times 10^2$
Right apical	2.0×10^3	$<1.0 \times 10^2$
Right cardiac	3.3×10^2	5.0×10^2
Right intermediate	1.2×10^3	$<1.0 \times 10^2$
Right diaphragmatic	5.0×10^2	3.3×10^2

*Impinger sample yielded 3.1×10^4 CFU/ml.

**Impinger sample yielded 9.6×10^3 CFU/ml.

***Sampling procedure could not detect *P. haemolytica* at a concentration of less than 1.0×10^2 CFU/gm of tissue.

TABLE 4 -- Number of *Pasteurella haemolytica* Recovered From Calf Lungs at Twenty-four Hours Post-aerosol Exposure

Lung Site	Non-exposed Calf (509)* (CFU/gm of Tissue)	Exposed Calf (507)** (CFU/gm of Tissue)
Left apical	1.5×10^3	$<1.0 \times 10^{2***}$
Left cardiac	1.3×10^4	$<1.0 \times 10^2$
Left diaphragmatic	$<1.0 \times 10^2$	$<1.0 \times 10^2$
Right apical	3.3×10^2	$<1.0 \times 10^2$
Right cardiac	2.0×10^2	$<1.0 \times 10^2$
Right intermediate	$<1.0 \times 10^2$	$<1.0 \times 10^2$
Right diaphragmatic	$<1.0 \times 10^2$	$<1.0 \times 10^2$

*Impinger sample yielded 1.2×10^5 CFU/ml.

**Impinger sample yielded 5.5×10^4 CFU/ml.

***Sampling procedure could not detect *P. haemolytica* at a concentration of less than 1.0×10^2 CFU/gm of tissue.

Harvest of Pulmonary Macrophages

Nasal and tracheal swabs done on the calves in this set of experiments resulted in recovery of *P. haemolytica* from the nasal and tracheal passage of LW10 prior to the first aerosolization procedure. *P. haemolytica* was not isolated from any other swabbing procedure in this set of experiments. Serological tests did not indicate viral activity in the calves of this set of experiments as indicated by negative or low (1:4) antibody titers during the duration of the experiment. Agglutination reactions indicated agglutinating antibodies against *P. haemolytica* in serum samples from LW10 and LW11 taken 10 days after the second aerosol exposure to *P. haemolytica*. Elevated temperatures were not noted in calves LW10 and LW11 following either experimental exposure to *P. haemolytica*.

Immunization of calf LW10 was accomplished by exposing it to an aerosol mist of *P. haemolytica* originating from a suspension of 1.5×10^9 CFU/ml and 1.4×10^9 CFU/ml respectively for the first and second aerosolization. Fifteen second impinger samples assaying the air this calf was inhaling yielded 2.1×10^5 and 6.8×10^4 CFU/ml respectively.

Calf LW11 was aerosolized with a *P. haemolytica* suspension containing 3.2×10^9 and 2.4×10^9 CFU/ml respectively for the 2 aerosols. Impinger samples of the air within the plastic bag yielded 7.8×10^5 and 1.5×10^5 CFU/ml respectively.

Pulmonary macrophages were harvested from the lungs of 11 calves, 2 of these were calves experimentally exposed to *P. haemolytica*. Data contained herein pertains to cells harvested from 2 non-exposed and 2 exposed calves.

Approximately 1.01×10^8 (range 3.2×10^7 to 3.1×10^8) respiratory cells were lavaged from each pair of lungs. Based upon Sano stain, the

population of cells were 98% mononuclear cells with a morphology of macrophages (Fig 1). Contaminating cells were lymphocytes, polymorphonuclear leucocytes, epithelial cells and in some instances a few red blood cells. There was no difference seen in the number of cells isolated from the exposed lung as opposed to the non-exposed lung.

Using McCoy's 5A medium macrophages were easily cultured and maintained on glass surfaces with attachment and marked cytoplasmic spreading (Fig 2). After 24 hours in tissue culture monolayers, these cells readily adhered to the surface of the glass, when washed with warm McCoy's medium supplemented with 20% FBS.

Bacterial Uptake by Pulmonary Macrophages

Approximately 30 to 40% of the pulmonary macrophages incubated with zymosan contained light to darkly staining red vacuoles when stained with PAS stain indicating the uptake of zymosan. Wells incubated with acridine orange showed better than 98% of the pulmonary macrophages to be viable at the time of incubation (Fig 3).

Attempts to induce phagocytosis of ^{14}C labeled *P. haemolytica* by bovine pulmonary macrophages were unsuccessful. Pulmonary macrophages from non-exposed and exposed lungs, when incubated with ^{14}C labeled *P. haemolytica in vitro*, showed no indication of phagocytosis as measured by lack of ^{14}C uptake. This finding was consistent throughout each set of reactions using antibody coated and non-coated bacteria. With the macrophages from the non-exposed lung (Table 5) the mean background counts of the lysate were 26.5 (range 25-30) and 26.0 (range 24-28) respectively for the two sets of experiments. (In each set of experiments at least 8 samples were taken from each experimental condition.)

Monolayers incubated with *P. haemolytica* which had been incubated

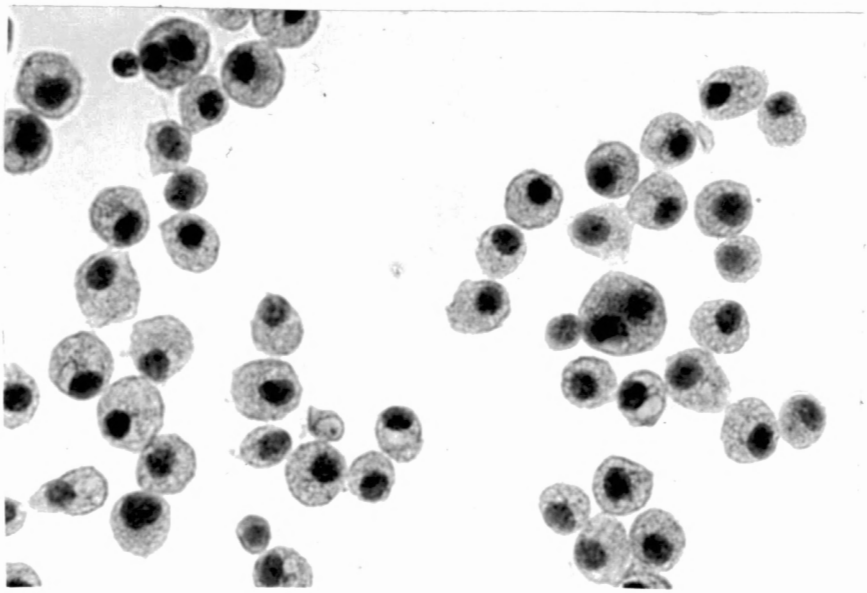

Fig 1 -- Bovine pulmonary macrophages harvested by pulmonary lavage, stained with Sano stain. Population predominately mononuclear cells. x 500.

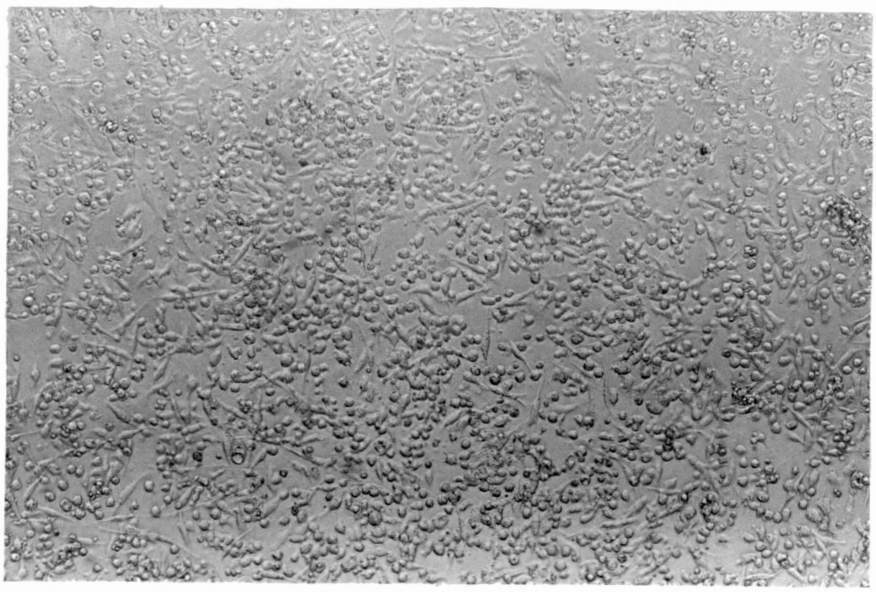

Fig 2 -- Twenty-four hour culture of bovine pulmonary macrophages grown on Lab Tek chamber slides in McCoy's 5A medium supplemented with 0.03% v/v L-glutamine and 20% FBS. Cytoplasmic spreading is prominent. x 400.

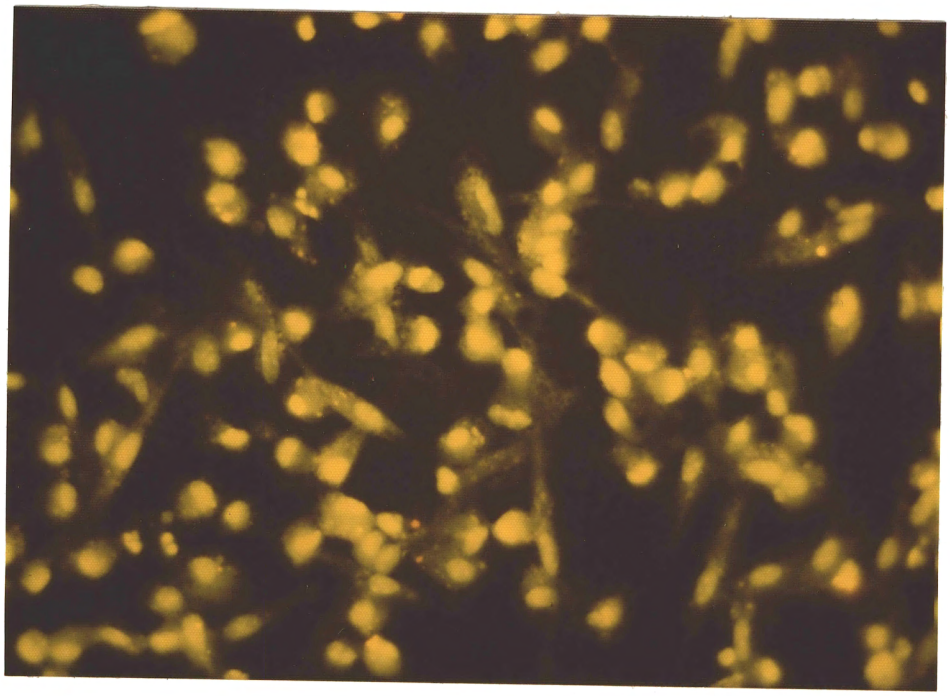

Fig 3 -- Pulmonary macrophages in culture 24 hours. Incubated 30 minutes with acridine orange (1:100). Green cells are viable, red cells are dead. x 800.

TABLE 5 -- Measurement of Phagocytic Activity of Pulmonary Macrophages Isolated From Lungs of Calves Not Exposed to an Aerosolized Mist of *Pasteurella haemolytica*

Sample	cpm/ml of Macrophage Lysate	
	Calf LW8	Calf LW9
Macrophages alone	26.0	26.5
Macrophages and *P. haemolytica*	26.7	27.0
Macrophages and *P. haemolytica* incubated with exposed sera	28.2	28.6
Macrophages and *P. haemolytica* incubated with non-exposed sera	33.1	27.3

with sera from a non-exposed calf had counts of 28.6 (range 22-41) and 28.2 (range 25-31) for LW8 and LW9 respectively.

Results from pulmonary macrophages isolated from exposed calves and incubated with coated and non-coated ^{14}C labeled *P. haemolytica* were similar to those of macrophages from non-exposed calves (Table 6). The range of these values were similar to those obtained from the non-exposed animals. These findings were consistent even when *P. haemolytica* was incubated with purified immunoglobulins from the lung (Table 7).

Fluorescent antibody staining of pulmonary macrophage monolayers incubated with *P. haemolytica* showed no evidence of phagocytosis under these experimental conditions during the 30 or 60 minute incubation time periods. This included macrophages incubated with *P. haemolytica* coated with the various immunoglobulin fractions.

Discussion

To coat *P. haemolytica* with serum antibodies or antibodies isolated from the bovine lung, a protein concentration of approximately 1.25 mg/ml was used. This concentration was based on results obtained by Reynolds[25] and employed here as a means of standardization. Reynolds found, using ^{125}I-labeled IgG antibody and ^{14}C labeled bacteria in a modified Farr test, that a protein concentration greater than 1.25 mg/ml provided an excess of antibody when the number of bacteria was 10^8/ml. Using this data the sera and purified immunoglobulin fractions from exposed and non-exposed calves were diluted to a protein concentration of 1.25-1.5 mg/ml and incubated with a bacterial suspension of 1.0×10^8 CFU/ml. These suspensions were further diluted 1:20 to dilute unreactive proteins to negligible amounts.

A number of procedures were tried in an effort to increase the in-

TABLE 6 -- Measurement of Phagocytic Activity of Pulmonary Macrophages Isolated From Lungs of Calves Exposed to an Aerosolized Mist of *P. haemolytica*

Sample	cpm/ml of Macrophage Lysate	
	Calf LW10	Calf LW11
Macrophages alone	29.25	20.50
Macrophages and *P. haemolytica*	23.75	35.25
Macrophages and *P. haemolytica* incubated with exposed sera	25.35	27.75
Macrophages and *P. haemolytica* incubated with non-exposed sera	26.45	32.62

TABLE 7 -- Measurement of Phagocytic Activity of Pulmonary Macrophages Toward ^{14}C Labeled *P. haemolytica* Previously Incubated with Purified Immunoglobulins from the Lungs of Exposed and Non-exposed Calves

	Immunoglobulins from a Non-exposed Calf cpm/ml of Macrophage Lysate	Immunoglobulins from an Exposed Calf cpm/ml of Macrophage Lysate
Pulmonary macrophages and *P. haemolytica* coated with purified IgA	40.87	30.87
Pulmonary macrophages and *P. haemolytica* coated with purified IgG	40.75	37.50

corporation of ^{14}C labeled amino acids into *P. haemolytica*. The first procedure employed was a modification of the method described by Reynolds.[25] This modification involved incubating *P. haemolytica* in 25 mls of BHI broth supplemented with 1% horse serum and 50 µci of ^{14}C labeled amino acid mixture. After 24 hours incubation there was a 8.5% incorporation of the isotope or approximately 1.2 x 10^5 CFU/cpm.

A second procedure was employed where *P. haemolytica* was grown in 25 mls of BHI broth supplemented with 1% horse serum for 4 hours, washed 2 times with 0.01 M PBS, pH 7.2, and suspended in a 0.0002 M glucose, 0.001 M MgCl, and 0.01 M PBS suspension, pH 7.2, containing 10 µci of ^{14}C labeled amino acid mixture. After 30 minutes incubation the bacteria were harvested by centrifugation, washed 5 times in PBS and resuspended in tissue culture medium. This procedure yielded a 14% incorporation of isotope for 2.9 x 10^4 CFU/cpm. By increasing the time the bacteria were in contact with the ^{14}C labeled amino acids to 60 minutes a 21% incorporation of the isotope was obtained for 1.0 x 10^4 CFU/cpm. By diluting the BHI broth medium 1:2 with distilled water a 35% incorporation could be obtained or 3.1 x 10^2 CFU/cpm. This dilution diluted the available amino acid pool and increased the competition for ^{14}C labeled amino acids. By having a ratio of 3.1 x 10^2 CFU/cpm meant that to have 1 cpm/ml increase in the macrophage lysate approximately 310 bacteria would have to be phagocytized. As there was approximately 5.0 x 10^5 to 1.0 x 10^6 macrophage per well and 30-40% of these were capable of phagocytizing zymosan, then approximately 1.5 x 10^5 to 4.0 x 10^5 macrophages were capable of phagocytosis. If each of these macrophages would have phagocytized 1 microorganism then it would have been possible to have ^{14}C counts of 500 to 1,000 cpm/ml of lysate. This was not seen under any of the experimental conditions employed. In-

stead counts of 20-50 cpm were found which were consistent with background counts indicating that at best less than 4% of the pulmonary macrophages were engaged in phagocytizing ^{14}C labeled *P. haemolytica*.

The pulmonary clearance study demonstrated that *in vivo* viable *P. haemolytica* is readily cleared from the lung and that this clearance may be enhanced by previous exposure. Even the 24 hour PBS calf that had extensive pulmonary edema was successful in reducing the number of viable *P. haemolytica*. The fact that these calves had a current or recent viral infection is indicated by the high titers against BVD virus. It is not known what effect this viral infection had on the health of the calves or clearance of *P. haemolytica*.

Post-aerosolization temperature increases were seen in calves exposed to *P. haemolytica* for the first time in the clearance study. After the second exposure this increase in temperature was not seen nor were there elevated temperatures seen in calves LW10 and LW11 following their experimental exposure to *P. haemolytica*. Also calf 501 became lame shortly after its first experimental exposure to *P. haemolytica* and remained so throughout the duration of the experiment. *P. haemolytica* was isolated from the lame, swollen joint during necropsy.

BVD virus has been reported to be an immunosuppressant. The data presented here indicate that in the presence of this viral activity previous exposure to *P. haemolytica* still enhanced pulmonary clearance. The mechanism of this clearance is unknown. *In vitro* studies employed here indicate that pulmonary macrophages do not phagocytize *P. haemolytica*, at least *in vitro*.

Ando et al[24] incubated pulmonary lavage fluids with their rabbit pulmonary macrophage and found they had an increase in phagocytic activity. Ando isolated IgG from his lavage fluid and found that this was the com-

ponent of the fluid that enhanced phagocytosis. In the experiments described in this paper, whole pulmonary lavage fluids were not incorporated into the macrophage growth medium. However, pulmonary IgG demonstrated to coat *P. haemolytica* was used by coating *P. haemolytica* before exposing it to the macrophages. This did not enhance phagocytosis.

Burrell[26] and Henney[27] have reported the presence of cell mediated immunity in the respiratory tract and Burrell[26] demonstrated immunization of the guinea pig respiratory tract with *Thermoactinomyces vulgaris* enhances a cell mediated immune response. Details of this aspect of pulmonary clearance have not been investigated in the bovine lung. However, it is reported that BVD infections can result in lymphopenia by attacking the lymphoid system.[28]

Critical problems associated with the harvest of the pulmonary macrophages are obtaining lungs free of ruminal content and blood. It was found in these experiments that aspiration of ruminal contents could occur if the calf was not hung head down within 3 minutes of death. A sterile cotton plug inserted into the trachea at the time the calf was hung insured the recovery of clean lungs. Ruminal content contamination of LWll prevented lavage of the total lung. Instead only 1 lobe, the right diaphragmatic lobe, was lavaged. Cutting the trachea during exsanguination can result in blood aspiration in some instances. Filling or evacuating the lungs too rapidly during the lavage process enhances the chances of damaging the respiratory epithelium. In these experiments it was found that a gradual filling, using not more than 1.5 L of fluid per filling at 2 PSI resulted in no or minimal red blood cells in the lavage fluid. Harvesting of the pulmonary macrophages by the procedure described here resulted in the recovery of approximately 2.0×10^4 pulmonary macrophages/ml of pulmonary lavage fluid. Centrifugation at 500 x g and 22°C minimizes

the stress placed on these cells and enhances their survival *in vitro*. Centrifugation at 1,000 x g and 4°C result in greater cell loss during medium changes.

Cell populations exceeding 4.0×10^6 cells per 162 mm of glass surface resulted in clumping of the macrophages and they were more easily detached from the surface during medium changes and washing procedures. The use of McCoy's 5A medium supplemented with 0.03% v/v L-glutamine and 20% FBS enhanced the maintenance of pulmonary macrophages *in vitro*. Initial studies using Dulbecco's minimal essential medium supplemented with 20% FBS as a tissue culture medium resulted in sloughing of the monolayer during the washing procedure employed after incubation with ^{14}C labeled *P. haemolytica*. The use of this medium also did not promote the attachment and cytoplasmic spreading seen with McCoy's 5A medium. It is not known why the pulmonary macrophages did not phagocytize *P. haemolytica*. Viability staining of the macrophages demonstrated that there was approximately 98% viability. The zymosan studies indicated that approximately 30-40% of the macrophages phagocytized zymosan.

Fox[19] using *Staphylococcus aureus* and bovine alveolar macrophages demonstrated that in a 30 minute incubation period the major portion of phagocytosis had taken place. In the writer's experiments phagocytosis could not be detected after 30 or 60 minutes using ^{14}C uptake or fluorescent antibody staining of the macrophage monolayer.

Reynolds[25] reported that many coated *Pseudomonas aeruginosa* that were not phagocytized by rabbit pulmonary macrophages during the allotted time were seen to line up adjacent to the macrophages. Fluorescent antibody examination of the macrophage monolayers employed here did not show this phenomenon to occur. These results suggest that the pulmonary macrophages may not be the primary defense mechanism in the lung in relation to *P.*

haemolytica infections. It is realized that these results are *in vitro* attempts to induce phagocytosis of *P. haemolytica* by pulmonary macrophages. Perhaps *in vivo* phagocytosis might occur where a total complement of resistant mechanism might interplay.

However, Friend[29] reported *P. haemolytica* to have a cytotoxic factor that could kill pulmonary macrophages upon contact. No information was given as to the nature of this cytotoxic factor. Acridine orange studies testing viability of the macrophages were not done following incubation with *P. haemolytica*. Perhaps this would have shed some light into the failure of the macrophages to phagocytize *P. haemolytica*.

Keiss[30] demonstrated that *P. haemolytica* possess an appreciable amount of endotoxin (12 to 25% of dry weight) which has a potency comparable to that of *Salmonella* species in the induction of hemorrhagic necrosis of skin and that systemic responses to the endotoxin occur only after the toxin reaches the pulmonary vascular bed regardless of injection sites.

Endotoxins associated with Gram negative bacteria are known to produce vascular leakage.[28] Tikoff et al[32] reported increased pulmonary vascular constriction resulting in pulmonary edema in calves injected with endotoxin. Jensen[10] reported that he frequently found bacteria and exudate extending along infected lumen down into alveolar ducts. Panciera et al[11] reported extensive edema in the lungs of control calves challenged by intrapulmonary inoculation of *P. haemolytica*. Calf 509 in the clearance study was found to have moist firm lungs indicating edema 24 hours after the aerosol exposure to *P. haemolytica*. These findings suggest that bovine pneumonic pasteurellosis caused by *P. haemolytica* may be due to the presence of an endotoxin.

In calves previously exposed to *P. haemolytica* in which an immune state has been described[11] it is possible that this immune state is due

to the presence of antibodies directed against the endotoxin of *P. haemolytica*, a somatic antigen, in addition to antibodies against *P. haemolytica*. Evidence to support this hypothesis could be obtained by attempting *in vitro* phagocytosis of a killed washed culture of *P. haemolytica* endotoxin could involve work similar to that of Gilka et al[13] or Panciera et al[11] using isolated and purified endotoxin from *P. haemolytica*. Specificity of the IgA and IgG isolated from the exposed bovine lung toward the endotoxin might also support this hypothesis, particularly if it can be demonstrated that the specificity is against the protein moiety of the lipid A portion of the endotoxin.

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CHAPTER II

STUDY OF BOVINE PULMONARY RESPONSE TO *PASTEURELLA HAEMOLYTICA* II. SPECIFICITY OF IMMUNOGLOBULINS ISOLATED FROM THE BOVINE LUNG

Summary

A procedure is described for the isolation and purification of the immunoglobulins IgA and IgG from pulmonary lavage fluid from calves. These immunoglobulins were isolated from calves experimentally exposed to *Pasteurella haemolytica* via aerosolization and from non-exposed calves. The specificity of these fractions toward *P. haemolytica* was examined using an indirect flourescent antibody test and agglutination reactions. Specific antibody activity was detected in the IgA and IgG fractions from calves exposed to *P. haemolytica* and IgG fractions of non-exposed calves. None of the isolated immunoglobulins agglutinated *P. haemolytica*.

Introduction

In the past few years considerable knowledge has been gained concerning the antimicrobial activity of mucous secretions of the respiratory tract. To date the majority of this work has involved antiviral mechanisms and has dealt primarily with the upper respiratory tract.[1,2] In the development of these data it has been demonstrated that intranasal instillation of viral vaccines usually lead to the production of nasal antibodies with little or no serum antibodies.[3,4] Ogra and his associates demonstra-

ted that viral vaccines administered into the respiratory tract resulted in antibody production in the area of the respiratory tract in which the immunizing agents were deposited.[4] These results showed that antigen deposited in the nose stimulated antibodies only in the nasal secretions and not in bronchial secretions. Waldman found that particles with a mean diameter of 1.5 μm were not retained in the nasal passage but deposited in the lower respiratory tract, resulting in antibody production in the lower respiratory tract and not in the nasal passage.[5]

The antibody produced in the upper respiratory tract against viral immunogens has been shown to be secretory IgA.[6] Secretory IgA is known to neutralize some viral particles and to prevent their attachment to the epithelium.[7] In viral infections it has been demonstrated that nasal antibody titers tend to increase earlier in individuals with a higher base line concentration of IgA in nasal secretions and that this phenomenon may be important in control of infection and prevention of illness.[15]

Less is known about antibacterial immune mechanisms of secretory IgA in the secretions of the respiratory tract.[8] In the bovine the predominant immunoglobulin in upper respiratory mucous secretions is secretory IgA.[8-12] IgG_1 is the predominant IgG subclass in these secretions with small amounts of IgG_2 also being present.[12-14] There is no IgM found in these secretions.[13]

In the feedlot industry respiratory diseases are a major problem, accounting for 40 to 80% of all cattle disease.[16] Viral infections represent a significant factor in these diseases processes.[17] However, the majority of these viral infections may go unnoticed or be mildly symptomatic.[17] A more serious consequence of these infections is the secondary invasion by the bacteria *Pasteurella multocida* and *Pasteurella haemolytica*.[19-24]

The development of an efficient means of immunizing the lower respiratory tract against these bacteria could result in a considerable savings to the feedlot industry. To determine the effectiveness of an immunizing agent requires an efficient means of monitoring the immune response to it. One way of monitoring this immune response is to measure the specificity of the immunoglobulins isolated from the bovine lung to the immunizing agent. The purpose of this paper is to describe a method of isolating, purifying and evaluating the specificity of immunoglobulins obtained from bovine lungs exposed to an aerosolized mist of *P. haemolytica* and from non-exposed lungs.

Materials and Methods

Preparation of *Pasteurella Haemolytica*

Pasteurella haemolytica used in these experiments has been described in detail elsewhere.[25] Briefly, *P. haemolytica* (isolate 12216) type 1 was isolated from the pneumonic lung of a calf and passaged twice in calves by intrapulmonic inoculations. For immunization of the calves lyophilized *P. haemolytica* was hydrated with 1.0 ml of sterile water and streaked for isolation onto brain heart infusion (BHI) agar plates[a] containing 1% yeast hydrolysate,[b] 1% horse serum[c] and 5% citrated bovine blood. After 24 hours incubation at 37°C in a candle jar typical *P. haemolytica* colonies were picked and used to inoculate additional BHI plates. After 24 hours incubation *P. haemolytica* was removed from the plates and suspended in phos-

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[a]Difco Laboratories, Detroit, Michigan.

[b]ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio.

[c]Pel Freez Biologicals, Inc., Rogers, Arkansas.

phate buffered saline, 0.01 M $NaPO_4$, 0.15 M NaCl, pH 7.2, (PBS) (Appendix) at a concentration of 1.0 x 10^9 CFU/ml.

Smears of *P. haemolytica* for indirect fluorescent antibody test (IFAT) were made by suspending a 20-24 hour colony of *P. haemolytica* in 1.0 ml of sterile distilled water. This suspension was diluted to approximately 1.0 x 10^6 CFU/ml and thoroughly mixed. One drop aliquots were dispensed to 22 mm^2 cover slips. Cover slips were air dried at room temperature for 24-48 hours before use.

P. haemolytica suspensions for adsorption of conjugated antibovine immunoglobulin antiserum[d] were prepared from a 20-24 hour culture grown on BHI agar plates. Cells from 1 heavily inoculated plate were suspended in 1.0 ml of PBS. This suspension was centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant fluid was discarded and the pellet was resuspended in 1.0 ml conjugated antibovine immunoglobulin antiserum diluted 1:20 in PBS.

Experimental Animals

Calves used in these experiments and the procedure used for their immunization have been described elsewhere.[25] Briefly, the calves were weaner age (300-500 pound) beef bred calves of both sexes. Two calves, designated LW10 and LW11, were exposed to an aerosol mist of *P. haemolytica* (1.0 x 10^9 CFU/ml) for 15 minutes 2 times at a 1 week interval. Two weeks after the second aerosolization the calves were killed and exsanguinated. Two non-exposed calves, LW7 and LW8, were exposed to an aerosol mist of PBS. Lungs from a third control calf, LW9, were obtained from a local abattoir at the time of the calf's death.

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[d]Miles Laboratories, Elkhart, Indiana.

Purification of Immunoglobulins

From Pulmonary Secretions

Lavage of the lungs was performed as previously described in a companion paper.[25] Briefly, the lungs were removed from the experimental animals within minutes after the animal was killed. Within 45 minutes after the death of the calves the lungs were lavaged by gently filling the lungs with sterile Rinaldi[26] salt solution (R-saline) containing 0.2% versene.[e] The lungs were filled and evacuated 3 times using 2 PSI positive and negative pressure respectively. The lavage fluid (approximately 2.0-2.5 L, 800 mls for LW11) was collected in a siliconized filtering flask. Pulmonary macrophages and contaminating cells were sedimented at 500 x g for 10 minutes at 22°C.

The resultant supernatant fluid was stored at 4°C for approximately 4 hours. Purification of the immunoglobulins from the supernatant fluid was accomplished using a modification of the technique described by Harboe[27] for purifying immunoglobulins from sera (Fig 1). These modifications involved adjusting the pH of the supernatant fluid (SF1) to pH 5.0 by slow addition of concentrated acetic acid with continuous stirring. The solution was allowed to stand for 30 minutes and the resultant precipitate (P1) sedimented by centrifugation at 16,000 x g for 20 minutes at 4°C. The precipitate (P1) was resuspended in 5.0 mls of PBS and stirred for 60 minutes at 25°C. This was followed by centrifugation at 20,000 x g for 20 minutes at 4°C. The supernatant fluid (SF2) from the resuspended precipitate (P1) was added to the previous supernatant fluid (SF1) and concentrated to approximately 5.0 mls at 4°C with an ultrafiltration appar-

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[e] Fisher Scientific, Dallas, Texas

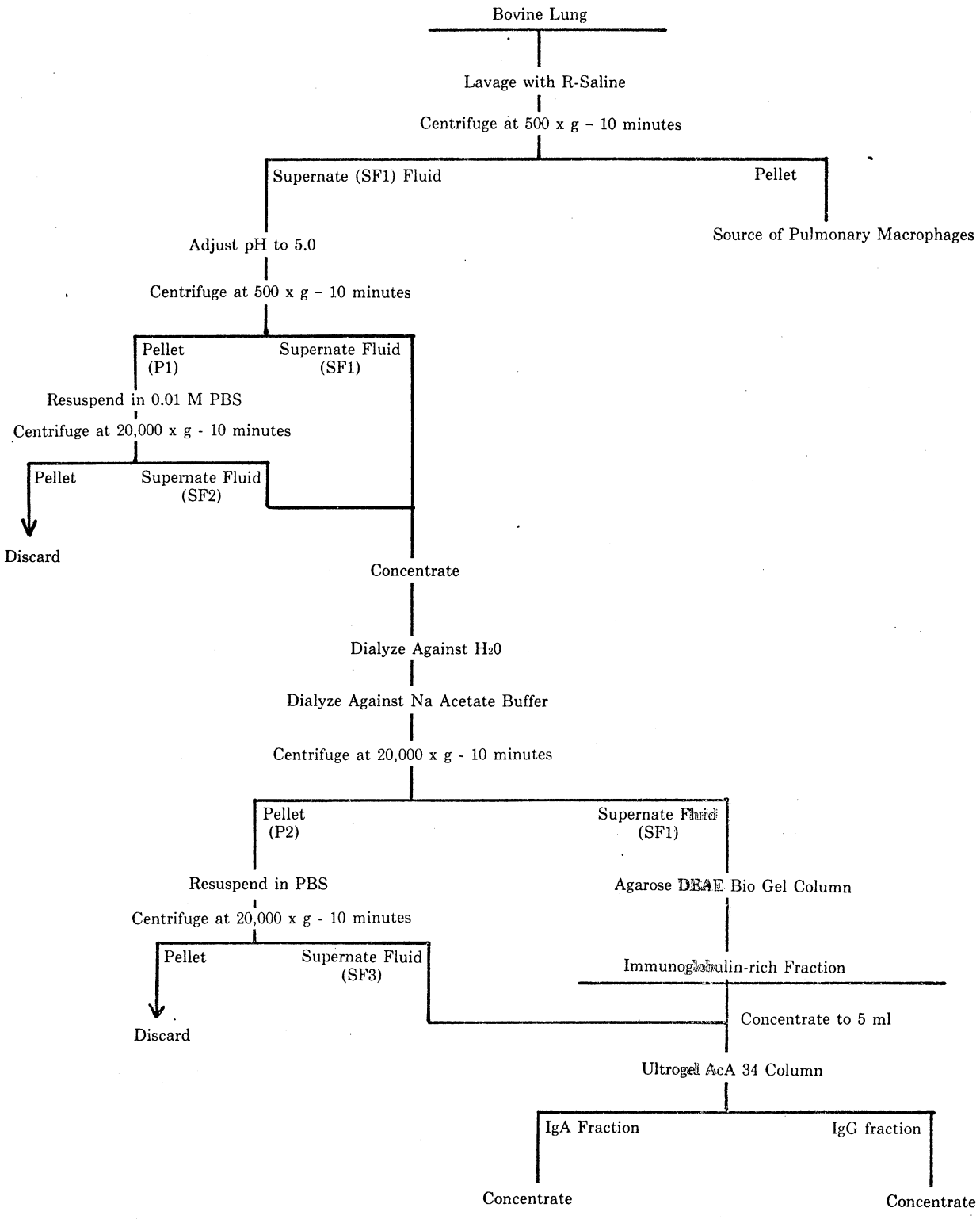

Fig 1 -- Isolation of immunoglobulins from pulmonary lavage fluids.

atus[f] capable of retaining molecules with a molecular weight exceeding 30,000 daltons.

The concentrated solution was dialyzed in distilled water at 4°C for 12 hours (1 change) and then in 0.035 M Na acetate buffer, pH 5.0 (Appendix) for 12 hours at 4°C. The precipitate which formed during dialysis was removed by centrifugation at 20,000 x g for 20 minutes at 4°C. The pellet was resuspended in 5.0 mls PBS, and stirred for 60 minutes at 25°C. This suspension was clarified by centrifugation at 20,000 x g for 20 minutes at 4°C. The supernatant fluid (SF3) from the resuspended pellet was decanted and held at 4°C.

The concentrated dialyzed supernatant fluid was applied to a DEAE agarose[g] ion exchange column (2.5 x 16 cm), equilibrated with 0.035 M Na acetate buffer, pH 5.0. The sample was eluted from the column with the same buffer at 20 ml/hr. using a peristalic pump.[h] Four ml fractions were obtained using a fraction collector[i] in the drop count mode (95 drops/tube). The absorbance of the eluent was monitored at 280 nm using an absorptometer and recorder.[j] Elutions were performed at room temperature (23°C). The column was washed with the starting buffer until absorbancy returned to base line. Once the absorbancy had returned to base line, the column was eluted with high ionic strength buffer (0.071 M Na acetate buffer, pH 5.0, 1.0 M NaCl) to remove proteins adsorbed to the column.

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[f]Model 402 and 52 stirred cells, with PM30 membrane, Amicon Corp., Lexington, Massachusetts.

[g]DEAE Bio-Gel A, Bio Rad, Richmond, California.

[h]Varioperpex Model 12000, LKB, Rockville, Maryland.

[i]ISCO Model 1200, ISCO, Lincoln, Nebraska.

[j]LKB Unicord II, LKB, Rockville, Maryland.

Immunoglobulin contents of the fractions were determined by a qualitative double immunodiffusion procedure. This procedure was performed in a 1.0 to 1.5 mm layer of 1% agarose[k] in PBS on a 84 x 94 mm glass slide prepared according to the method of Weeke.[28] Three parallel rows of 11 wells (4 mm in diameter, 7 mm center to center) were cut and aspirated in the agar. The center row of wells in each pattern was incubated with 10 µl of a 1:5 dilution of rabbit antibovine IgG (heavy and light chain specific).[l] The outer rows contained 10 µl of eluate from sequential tubes. A duplicate immunodiffusion test was done using 10 µl of a 1:5 dilution of rabbit antibovine IgA[b] in the center row. Tubes containing the immunoglobulin fractions were pooled, approximately 50 mls, and concentrated to a 6 ml volume by ultrafiltration as done previously at 4°C. The supernatant fluid obtained from the dialysate precipitate (SF3) and held at 4°C was added to the immunoglobulin-rich fraction prior to concentration. The concentrated immunoglobulin-rich fraction was adjusted to pH 7.0 with 0.2 M K_2HPO_4 and applied to an Ultrogel AcA 34 column[m] (2.6 x 88 cm), approximately 12 mls, by layering the sample between the eluent and the gel bed using a peristalic pump. Layering was enhanced by adding sucrose (5% w/v) to increase the density of the immunoglobulin solution prior to placing it on the gel bed. The Ultrogel AcA 34 column had been washed with 0.01 M $NaPO_4$, 0.15 M NaCl, pH 7.2 (PBS) prior to use. The sample was eluted from the column with PBS, delivered at 20 ml/hr. by a metering pump.[n] Five ml fractions were collected on a time interval basis (15 min./

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[k] MCI Seakem, Microbiological Associates, Bethesda, Maryland.

[l] Cappel Laboratories, Downington, Pennsylvania.

[m] LKB, Rockville, Maryland.

[n] ISCO Model 312, ISCO, Lincoln, Nebraska.

fraction). Absorbancy of the eluate was monitored at 280 nm using an absorptometer and recorder.

Immunoglobulin contents of fractions were determined by a qualitative assay using double immunodiffusion technique as described above. Fractions containing IgA or IgG were concentrated to 5 mls and stored at 4°C until used.

Protein concentrations of the isolated immunoglobulins were determined by absorbancy at 280 nm on a spectrophotometer[o] assuming an $E^{1\%}_{280}$ of 14.0.[29]

Quantitative Immunoglobulin Assay

Immunodiffusion test kits[k] were utilized to determine bovine IgA and IgG immunoglobulin levels by single radial diffusion.[30] Monospecific antisera to bovine immunoglobulins of rabbit origin were incorporated into the agar medium. Capillary pipets were used to fill the wells. The plates were incubated 24 hours at room temperature in a moist atmosphere. The radius of the precipitate formed was dependent on the quantity of specific immunoglobulins present. Purified immunoglobulin fractions were compared to precipitates formed by monospecific antibovine immunoglobulin reference sera.

This technique was also used to monitor the purification procedure by assaying the various fractions obtained at each step for the presence of IgG, IgA and IgM.

Indirect Fluorescent Antibody Test

The indirect fluorescent antibody test (IFAT), method described by

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[o]Zeiss PMQ II Spectrophotometer, Carl Zeiss, West Germany.

Potgieter,[31] was employed to assay the specificity of the immunoglobulins purified toward *P. haemolytica*. This procedure involved flooding bacterial smears with a purified immunoglobulin fraction. After 30 minutes incubation in a humid chamber at 37°C the smears were washed with PBS, rinsed with distilled water and dried at room temperature. These smears were then flooded with adsorbed conjugated antibovine immunoglobulin antiserum[b] diluted to 1:20 in PBS. After 30 minutes incubation the smears were washed with PBS, rinsed with distilled water and air dried. The conjugated antiserum was adsorbed with viable *P. haemolytica* (prepared as described previously) for 18 hours at 4°C, centrifuged at 20,000 x g for 10 minutes at 4°C and filtered through a 450 nm membrane filter.[p] Controls consisted of smears of *Pasteurella multocida* and *Pseudomonas aeruginosa* prepared in the same manner as *P. haemolytica* and direct fluorescent antibody staining of these 3 smears.

Agglutination Reaction

P. haemolytica for agglutination reactions were grown overnight on BHI agar plates. One *P. haemolytica* colony was removed from the 18-24 hour plate and mixed thoroughly with 1 drop of serum or immunoglobulin preparation on a glass slide. Agglutination occurred within 15 seconds for those samples containing agglutinating antibodies against *P. haemolytica*. *Pseudomonas aeruginosa* was used as a bacterial control and serum from a non-exposed calf was used as a serum control as was PBS.

Results

The data presented here represents results obtained from 3 non-

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[p]Millipore Filter Corporation, Bedford, Massachusetts.

exposed and 2 exposed calves. Four to 4.5 liters of saline solution were instilled during the lavage of each lung. Approximately half of this volume could be recovered. The exception to this was with LW11 where ruminal contents were found to be contaminating the major airways in the lung. In this lung only the airways leading into the right diaphragmatic lobe were free of ruminal contents, therefore, only this lobe was lavaged.

By lowering the pH of the washings to 5.0 after removal of the macrophages, lipoproteins and other contaminating proteins were precipitated and removed by centrifugation. Assay of the solubilized precipitate (P1) did not reveal the presence of immunoglobulins in this fraction. Remaining proteins including immunoglobulins were concentrated to approximately 5 mls. Due to the large initial volume, concentration was initiated in the 402 stirred cells and completed in the 52 stirred cells. Dialysis against distilled water followed by dialysis against 0.035 M Na acetate buffer resulted in the formation of a light precipitate. This precipitate (P2), when solubilized and assayed for the presence of immunoglobulins using immunodiffusion test kits, was found to contain trace amounts of IgA and IgG. The majority of the IgG and IgA remained in solution. A typical elution profile from separation of the soluble immunoglobulin fraction on DEAE agarose is shown in Figure 2.

Immunoglobulins, as determined by double immunodiffusion were found to be in fractions 7-22. The IgA fraction was eluted first in fractions 7-12 (Fig 3). IgG eluted slightly later between fractions 7-19 (Fig 4). As the antibovine IgG antibody was heavy and light chain specific it was difficult to distinguish IgG from IgA in this particular double immunodiffusion assay. Immunodiffusion analysis of the eluate eluted with the high ionic strength buffer did not reveal the presence of immunoglobulins to these fractions even after concentration. Thus, virtually all of the

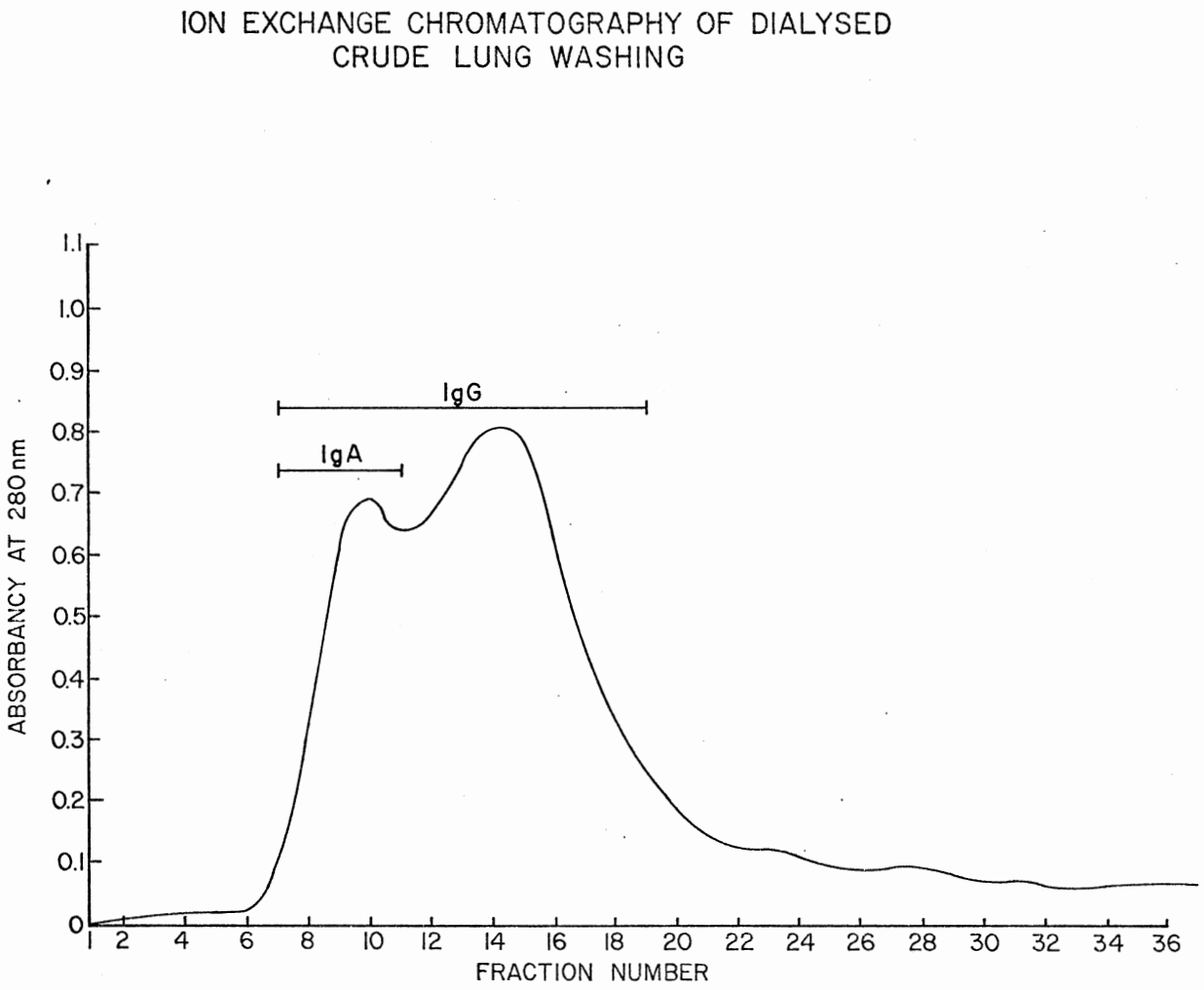
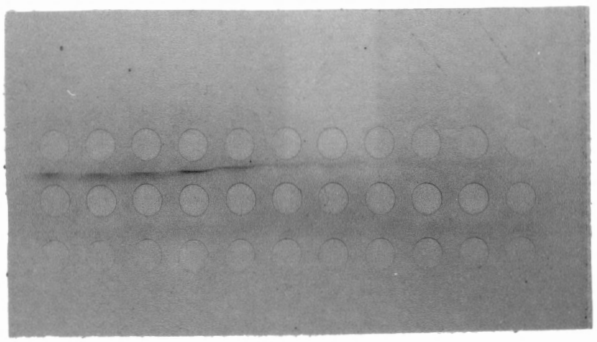


Fig 2 -- Elution profile of crude concentrated lung washing from a DEAE agarose column (2.6 x 16 cm) eluted with 0.035 M Na acetate, pH 5.0. Four ml fractions were collected and monitored at 280 nm. Immunoglobulin peaks determined by double immunodiffusion.

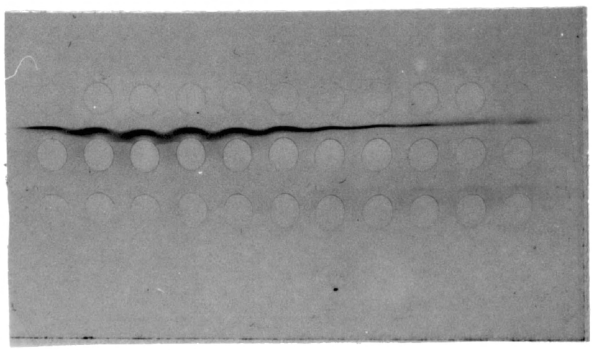


Bovine Immunoglobulin, 20 µl

Antibovine IgA, 10 µl

Bovine Immunoglobulin, 20 µl

Fig 3 -- Double immunodiffusion analysis of DEAE elution profile. Antibody (center well) is rabbit antibovine IgA (monospecific) diluted 1:5 10 µl per well. Antigen is elution fractions 7-17, left to right, top row; 28-18 bottom row, 20 µl per well. IgA was found in fractions 7-12.



Bovine Immunoglobulin, 10 µl

Antibovine IgG, 10 µl

Bovine Immunoglobulin, 10 µl

Fig 4 -- Double immunodiffusion analysis of DEAE elution profile. Antibody (center well) is rabbit antibovine IgG (heavy and light chain specific) diluted 1:5, 10 µl/well. Antigen is elution fractions, left to right, 7-17, top row; 28-18, bottom row, 20 µl per well. IgG was found between fractions 7-19.

immunoglobulin eluted from the column with the starting buffer.

A typical elution profile from the molecular sieve column is shown in Figure 5. IgA fractions eluted first coming off in fractions 40-47 as determined by double immunodiffusion (Fig 6). IgG fractions eluted from the column in fractions 50-63 (Fig 7). The fractions containing each immunoglobulin were pooled and concentrated to approximately 5 mls.

Analysis of the concentrated IgA and IgG fractions using immunoelectrophoresis and radial immunodiffusion indicated both fractions were essentially pure.[q] In the IgG fraction approximately 95% of the IgG was of subclass IgG_1 with the remainder of IgG_2.

The concentrations of purified antibodies as determined by radial immunodiffusion kits are shown in Table 1. From each lung the concentrations of IgA were found to be greater than the concentration of IgG. This is true even of LW11 which was taken only from the right diaphragmatic lobe.

Each immunoglobulin fraction isolated was assayed for specificity against *P. haemolytica* using a slide agglutination reaction and indirect fluorescent antibody test (IFAT). None of the immunoglobulins isolated gave a positive agglutination reaction when tested with *P. haemolytica* (Table 2). IgA isolated from the lungs of the non-exposed calves (LW7, LW8 and LW9) showed no specificity toward *P. haemolytica* by IFAT. The IgA fractions from LW10 and LW11 (exposed calves) both gave positive IFAT reactions. The IgG fraction from LW7 did not coat *P. haemolytica* as determined by IFAT; whereas the IgG fraction from LW8, LW9, LW10 and LW11 did coat *P. haemolytica*. The reactions seen with IgG from LW10 and LW11 were strongly positive. A 4+ reaction is shown in Figure 8.

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[q]Personal communication, P.K. Chung, Miles Lab., Elkhart, Indiana.

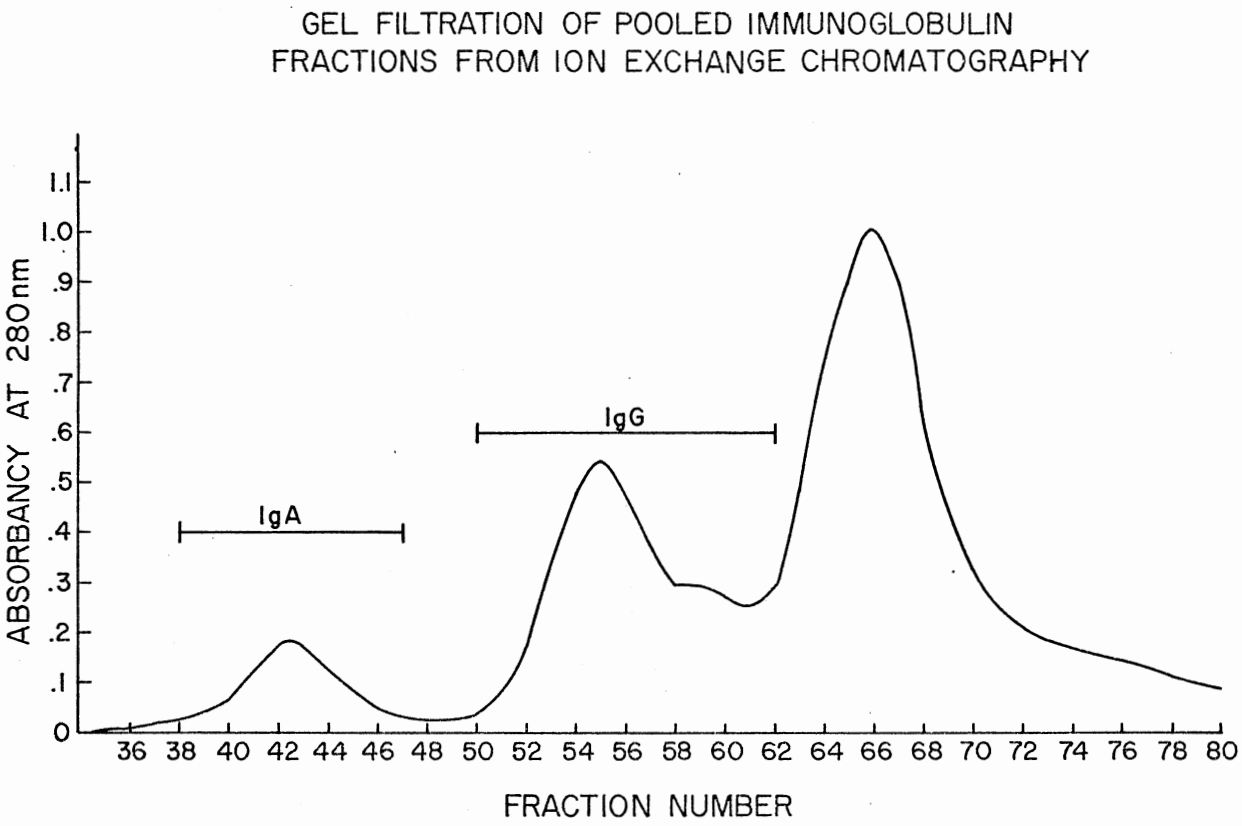
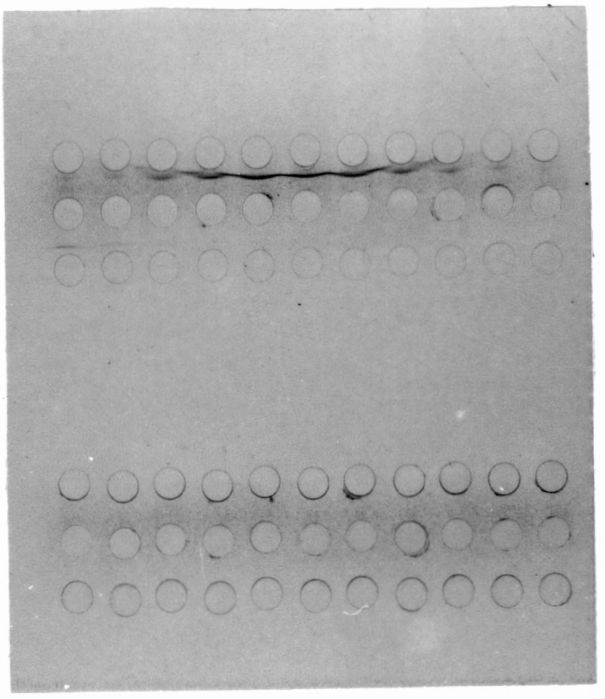


Fig 5 -- Elution profile of DEAE immunoglobulin-rich eluate concentrated and applied to Ultrogel AcA 34 column (2.6 x 88 cm). Sample eluted with 0.01 M PBS, pH 7.2. Five ml fractions were collected with absorbancy being monitored at 280 nm. Immunoglobulin peaks determined by double immunodiffusion.



Bovine Immunoglobulin, 20 µl

Antibovine IgA, 10 µl

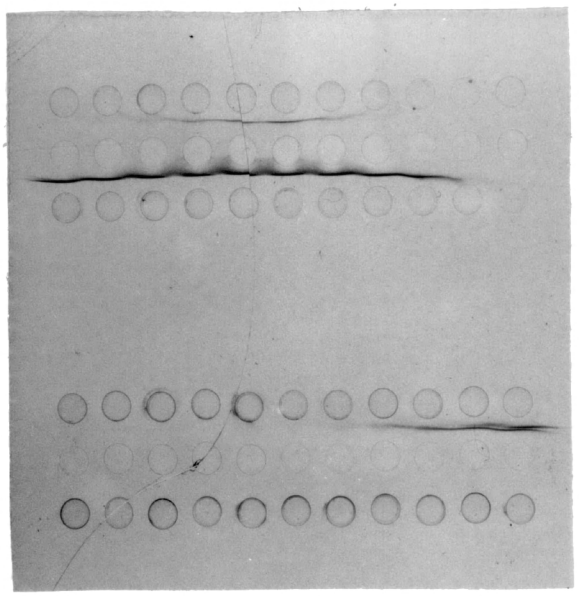
Bovine Immunoglobulin, 20 µl

Bovine Immunoglobulin, 20 µl

Antibovine IgA, 10 µl

Bovine Immunoglobulin, 20 µl

Fig 6 -- Double immunodiffusion analysis of Ultrogel AcA 34 elution profile. Antibody (center wells) is rabbit antibovine IgA (monospecific) diluted 1:5, 10 µl per well. Antigen is elution fractions 38-48, row 1; 49-59, row 2; 60-70, row 3; and 71-81, row 4, 20 µl per well. IgA antibodies were in fractions 40-47.



Bovine Immunoglobulin, 10 µl

Antibovine IgG, 10 µl

Bovine Immunoglobulin, 10 µl

Bovine Immunoglobulin, 10 µl

Antibovine IgG, 10 µl

Bovine Immunoglobulin, 10 µl

Fig 7 -- Double immunodiffusion analysis of Ultrogel AcA 34 elution profile. Antibody (center wells) is rabbit antibovine IgG (heavy and light chain specific) diluted 1:5, 10 µl per well. Antigen is elution fractions 38-48, row 1; 49-59, row 2; 60-70, row 3; and 71-81, row 4, 20 µl per well. IgG antibodies were in fractions 50-63.

TABLE 1 -- Immunoglobulin Concentrations of Immunoglobulins Isolated From Bovine Lung

Calf	IgA Fraction mg/ml	IgG Fraction mg/ml
LW7*	2.0	0.2
LW8*	1.0	0.19
LW9**	7.9	1.7
LW10***	15.0	1.8
LW11***	2.0	0.6

*Control calves not experimentally exposed to *P. haemolytica*.

**Control calf from abattoir.

***Calves experimentally exposed to *P. haemolytica*.

TABLE 2 -- Specificity of Pulmonary Immunoglobulins Toward *Pasteurella Haemolytica*

Calf	Agglutination *P. haemolytica*	Indirect Fluorescent Antibody Test *P. haemolytica*	*P. multocida*	*P. aeruginosa*
LW7*				
IgA	-	-	-	-
IgG	-	-	-	-
LW8*				
IgA	-	-	-	-
IgG	-	2+	-	-
LW9**				
IgA	-	-	-	-
IgG	-	2+	2+	-
LW10***				
IgA	-	4+	-	-
IgG	-	4+	±	-
LW11***				
IgA	-	3+	-	-
IgG	-	4+	+	-

*Control calves not experimentally exposed to *P. haemolytica*.

**Control calf from abattoir.

***Calves experimentally exposed to *P. haemolytica*.

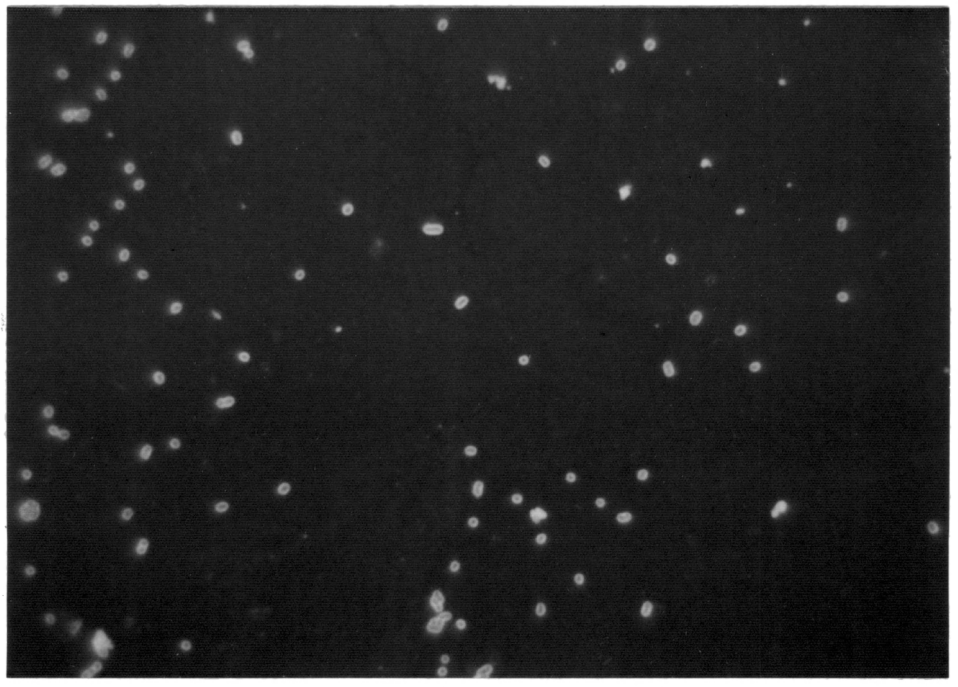


Fig 8 -- Fluorescence of *Pasteurella haemolytica* using indirect fluorescent antibody test. A *P. haemolytica* smear was incubated with IgG isolated from LW10. Conjugated rabbit antibovine immunoglobulins were applied to washed smears. IgG from LW10 coated *P. haemolytica*. x 800.

Each immunoglobulin fraction was also assayed for specificity toward *P. multocida* and *Pseudomonas aeruginosa* using IFAT. LW9 IgG and LW10 IgG both showed specificity toward *P. multocida*. IgA samples were negative against *P. multocida*. All samples were negative against *Pseudomonas aeruginosa*.

Immunoglobulin fractions that gave positive IFAT reactions against *P. haemolytica* were diluted to a common protein concentration and assayed. LW8 had a protein concentration of 260 µg/ml therefore each sample was diluted to this concentration. LW8 gave negative results when further diluted 1:2. LW10 IgA and LW10 IgG both gave positive reactions when diluted to 260 µg/ml (a dilution of 1:6.5 and 1:21.1 respectively). LW11 IgA and IgG also showed specificity toward *P. haemolytica* when diluted to this protein concentration (a 1.7 and 6.7 dilution respectively). When further diluted LW10 IgA had a titer of 1:16, LW10 IgG had a titer of 1:64. IgA and IgG from LW11 had titers of 1:8 and 1:64 respectively.

Sera from each calf, except LW9, were also assayed using the slide agglutination reaction and IFAT. Sera from the non-exposed calves were non-reactive toward *P. haemolytica* by the agglutination test. Sera from the exposed calves were negative pre-aerosolization but 10 days after the second aerosol both serum samples did agglutinate *P. haemolytica*. Sera from all the calves tested except LW7 coated *P. haemolytica* as demonstrated by IFAT.

Discussion

The method described for the isolation and purification of IgA and IgG from lavage fluids from the bovine lung has been successful in this laboratory. Radial immunodiffusion assay, using an IgM monospecific test kit, of samples throughout the purification procedure did not reveal the

presence of IgM in any fraction.

A number of laboratories have reported the results of studies of the distribution of immunoglobulins in bovine secretions.[9-14] These laboratories report that IgA and IgG are the major immunoglobulins of bovine nasal secretions, with trace amounts of IgG_2 but no IgM. The purity of the IgA and IgG fractions obtained in this laboratory were confirmed by Dr. P. K. Chung at Miles Laboratory, Elkhart, Indiana using immunoelectrophoresis. Samples were sent to Dr. Chung when it was found that in using an anti-IgG radial immunodiffusion kit precipitin rings developed around the wells containing IgA fractions. Dr. Chung's analysis was that the bovine IgG lung washing contained mostly IgG_1 subclass with a slight amount of IgG_2, but no IgA. The IgA fraction contained pure IgA by immunoelectrophoresis and immunodiffusion. These results indicated that the Miles Laboratory IgG determination kit was not monospecific for IgG.

In the purification of these immunoglobulins the initial starting volume of 2.0-2.5 liters may be quickly concentrated to 5 mls in approximately 48-72 hours, at a pH of 5.0. Adjusting the pH to 5.0 prior to concentration resulted in the precipitation of lipoproteins. This resulted in a saving of 24 to 48 hours in concentration time without a detectable loss of IgA or IgG.

Quantitative analysis of the recovery of the individual fractions were not reported here due to the inherent variance found in radial immunodiffusion. Analysis by rocket electrophoresis could provide this information but was not employed here.

It is felt that the IgA isolated from the lungs was secretory IgA. IgA eluted through the molecular sieve column ahead of IgG and with clean separation. Monomeric IgA has a molecular weight of 160,000 daltons which is in the range of IgG (150,000 daltons). If IgA was in the monomeric form

it would have co-eluted with IgG or at least would have heavily contaminated it. Hach and Pahud[10] found that SIgA is a major component of most external secretions including saliva and nasal secretions. Duncan et al[12] supports these results and found that bovine serum IgA also had a molecular weight consistent with its dimeric form.

To date the author is not aware of work where IgA has been purified from bovine lungs. It has been found in this study that IgA is the major immunoglobulin in the lower respiratory tract of exposed and non-exposed calves. This is especially emphasized by the lavage of LW11 which was done only on the right diaphragmatic lobe, yet still had an IgA concentration better than 3 times that of IgG.

IgA isolated from the lungs of exposed calves coated *P. haemolytica*. IgA from lungs of non-exposed calves did not. Also, none of the IgA isolated coated *P. multocida*. On the other hand IgG from 2 exposed and 2 non-exposed calves coated *P. haemolytica*. IgG from the 2 exposed calves and 1 non-exposed calf coated *P. multocida*. The significance of this is not yet fully understood. It may be that IgA represents a more recent infection, whereas IgG represents previous exposure. Duncan and Thomson[32] found in their study that 5 out of 15 calves in their experiments had naturally occurring serum IHA antibodies against *P. haemolytica* indicating that antibodies against *P. haemolytica* are not uncommon.

The exact mechanism of action of IgA against bacteria is yet to be elucidated.[8] It is known that it is a more efficient agglutinator than is IgG.[33] This is possible due to the fact that in most respiratory secretions IgA is in the dimeric form and thus has 4 attachment sites rather than the 2 sites IgG has. Yet in these experiments IgA did not agglutinate *P. haemolytica*. IgA has also been shown to neutralize diphtheria toxin.[34,35] *P. haemolytica* has been shown to possess an endotoxin which is

phospholipid-polysaccharid protein in nature and makes up 12-25% of the dry weight.[36] However, the response of SIgA to *P. haemolytica* endotoxin is unknown. SIgA has been shown to reduce the ability of bacteria to attach to the mucosal surface.[37,38] This may be a significant factor in *P. haemolytica* infections as *P. haemolytica* appears to attach to or locate near the mucosal surface and multiply.[18] Secretory IgA has also been shown to inhibit the growth of lag-phase *Pseudomonas aeruginosa*.[39] In the presence of complement and lysosome SIgA has been shown to lyse some Gram negative bacteria.[16] Normal respiratory secretions possess lysosome,[39] but complement is noticably absent.[33] However, in an inflammatory state where there is vascular leakage, complement may be found in the increased secretions.[40]

Specific secretory IgA has also been shown in this work and by others[33] to coat certain bacteria. However, this coating does not appear to enhance phagocytosis as there is no evidence for IgA receptors on alveolar macrophages.[41] Instead this reaction may be primarily to block receptors on the bacterial cell surface and thus prevent their attachment and colonization on the mucosal surface.

Newhouse et al[40] have reported that IgA antibodies present in nasal and upper respiratory tract secretions is better correlated with resistance to infection than serum antibodies of any class. It is possible that this applies to the lower respiratory tract as well.

The amount of IgA and IgG in exposed lungs appeared to be considerably higher than the amount of IgA and IgG from non-exposed. (LW11 represents lavage of only 1 lobe compared to the lavage of the whole lung for the remaining calves.) The mechanism of action of these antibodies may be that they coat *P. haemolytica* and prevent its colonization of the mucosal surface. These antibodies may also be directed against *P. haemolytica* endotoxin and more specifically the lipid A protein moiety and thus

neutralize its toxicity. Further investigation needs to be done on the exact mechanism of action of these antibodies (IgA and IgG) against *P. haemolytica*.

Although the lavage procedures reported here were performed on lungs removed from the calf, it is possible that pulmonary lavages could be conducted on live animals. This would enable one to analyze the immunological changes that take place in relation to the immunization procedures per animal, and thus measure more accurately the actual immunological response of the bovine lung to *Pasteurella haemolytica*.

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SUMMARY

The data presented in this thesis demonstrates that a previous exposure to an aerosolized mist of *P. haemolytica* enhances the clearance of this organism from the bovine lung. The possible mechanisms of this clearance were studied *in vitro*. Attempts to induce phagocytosis of ^{14}C labeled *P. haemolytica* by pulmonary macrophages obtained by pulmonary lavage from lungs exposed to *P. haemolytica* and non-exposed lungs were unsuccessful. Prior coating of *P. haemolytica* with serum from exposed and non-exposed calves and IgA and IgG isolated from the lungs of these calves did not facilitate phagocytosis.

A technique for the isolation of immunoglobulins from bovine pulmonary lavage fluids is described. IgA and IgG antibodies isolated by the procedure described were found to be pure. Indirect fluorescent antibody test (IFAT) results showed IgA isolated from the lungs of a calf exposed to an aerosolized mist of *P. haemolytica* to coat *P. haemolytica*. IgA isolated from non-exposed calves did not coat *P. haemolytica*. IgG from 2 non-exposed calves and 2 exposed calves coated *P. haemolytica*. IgG from 1 non-exposed calf did not coat *P. haemolytica*. None of the antibody preparations agglutinated *P. haemolytica*.

This study indicates that an increase in clearance of *P. haemolytica* from the lungs of a calf previously exposed to this organism may not be due to pulmonary macrophages. Instead the increased resistance may be due to specific antimicrobial activities of immunoglobulins in secretion of the lower respiratory tract and more specifically to IgA.

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APPENDIXES

PHOSPHATE BUFFERED SALINE

(PBS)

0.01 M phosphate (K)

0.15 M NaCl

pH 7.2

Compounds	Amount per Liter
KH_2PO_4	449 mgs
K_2HPO_4	1.167 gms
NaCl	8.767 gms

ISOTOPE INCORPORATION MEDIUM

Compounds	Amount per Liter
KH_2PO_4	449 mgs
K_2HPO_4	1.167 gms
NaCl	8.767 gms
Glucose	36 mgs
MgCl	203 mgs
L-amino acid - ^{14}C (U) mixture*	30 µci

*New England Nuclear

R-SALINE WITH 0.2% VERSENE

Compounds	Amount per Liter
NaCl	8.00 gms
KCl	0.20 gms
$Na_3C_6H_5O_7 \cdot 2H_2O$	1.00 gm
$NaH_2PO_4 \cdot H_2O$	0.05 gms
$NaHCO_3$	1.00 gm
Glucose	1.00 gm
Versene	2.00 gms

Adjust pH to 7.10

Filter sterilize.

ACETIC ACID - Na ACETATE BUFFER*

0.035 M with respect to acetate

pH 5.0

ionic strength 0.05

acetic acid 0.021 M

Na acetate 0.05 M

Stock Solutions:

Solution A

1.0 M acetic acid

30.1 gms glacial acetic acid in 500 mls water

Solution B

2.0 M sodium acetate

136.1 gms $NaC_2H_3O_2 \cdot 3H_2O$ in 500 mls water

Buffer from Stock Solutions:

Solution A	21.0 mls
Solution B	25.0 mls

Dilute to 2 liters with distilled water

*Taken from Harboe, N. and Ingild, A. Immunization, Isolation of Immunoglobulins, Estimation of Antibody Titre. A Manual of Quantitative Immunoelectrophoresis. Chapter 23. ed. by N. H. Axelsen, J. Kroll and B. Weeke. Universitetsforlaget Oslo Norway.

VITA 2

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Thesis: A STUDY OF BOVINE PULMONARY RESPONSE TO *PASTEURELLA HAEMOLYTICA*. I. PULMONARY MACROPHAGE RESPONSE. II. SPECIFICITY OF IMMUNOGLOBULINS ISOLATED FROM THE BOVINE LUNG

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